

APPENDIX C

MARKED-UP COPY OF SUBSTITUTE SPECIFICATION

NATURAL KILLER CELL LINES AND METHODS OF USE

RELATED APPLICATION

This is a continuation-in-part of U.S. Patent Application No. 09/403,910
5 filed October 27, 1999 (now abandoned), which was a nationalized
application of PCT Application No. PCT/US98/08672 filed April 30, 1998,
which claims priority to U.S. Provisional Patent Application No. 60/045,885
filed April 30, 1997.

FIELD OF THE INVENTION

10 This invention relates to natural killer cells and their use in the
treatment of pathologies related to cancer or viral infections. Specifically, a
particular cell line, NK-92, and modifications thereof, are disclosed. These
cells are shown to be highly effective in the treatment of these pathologies.

BACKGROUND OF THE INVENTION

15 Certain cells of the immune system have cytotoxic activity against
particular target cells. Cytotoxic T lymphocytes (CTLs) are specifically
directed to their targets via antigen-derived peptides bound to MHC class I-
specific markers. Natural killer (NK) cells, however, are not so restricted. NK
cells, generally representing about 10-15% of circulating lymphocytes, bind
20 and kill target cells, including virus-infected cells and many malignant cells,
nonspecifically with regard to antigen and without prior immune sensitization
(Herberman et al., *Science* 214.24 (1981)). Killing of target cells occurs by
inducing cell lysis. MHC class restriction likewise is not involved. In these

ways the activity of NK cells differs from antigen-specific and MHC class-specific T cells, such as cytotoxic T lymphocytes. Use of NK cells in the immunotherapy of tumors and malignancies is suggested by these properties, since many tumors are MHC class I deficient and therefore do not attract CTL activity. Adhesion molecules may also be involved in the targeting of NK cells; for example, it is observed that the Fc γ receptor (CD16) is expressed on NK cells. NK cells are large granular lymphocytes which lack CD3, and in addition to CD16, also may express Leu19 (Lanier et al., *J. Immunol.* 136; 4480 (1986)).

10 NK cells are activated when exposed to cytokines such as interleukin-2 (IL-2), IL-7, IL-12, and interferons (Alderson et al., *J. Exp. Med.* 172:577-587 (1990); Robertson et al., *J. Exp. Med.* 175:779-788 (1992)). The resulting cells are called lymphokine activated killer (LAK) cells. The spectrum of target cells is altered in activated NK cells compared to nonactivated cells, 15 although the mechanism of killing may be identical or similar (Philips et al., *J. Exp. Med.* 164:814-825 (1986)).

More generally, killing activity in the cells of the immune system may be induced by treating a population of cells, such as peripheral blood mononuclear cells (PBMCs), with lymphokines. Such preparations contain 20 LAK cells. LAK cells may also be generated from autologous samples of peripheral blood lymphocytes. LAK cells have antitumor killing activity while having essentially no effect on normal cells. They appear to purge leukemia (Long et al., *Transplantation* 46:433 (1988); Xhou et al., *Proc. Am. Assoc. Cancer Res.* 34:469 (1993; abstract)), lymphoma (Schmidt-Wolf et al., *J. Exp. Med.* 174: 139 (1991); Gambacorti-Passerini et al., *Br. J. Haematol.* 18:197 (1991)) and neuroblastoma (Ades et al., *Clin. Immunol. Immunopathol.* 46:150 (1988)). NK cells, activated NK cells, and LAK cells are 25

distinguishable by their cell surface markers and by the identity of the target cells that they kill.

Activated and expanded (i.e., cultured to proliferate) NK cells and LAK cells have been used in both *ex vivo* therapy and *in vivo* treatment in patients with advanced cancer. Some success with *ex vivo* therapy has been
5 observed in bone marrow related diseases, such as leukemia, breast cancer and certain types of lymphoma. *In vivo* treatment may be directed toward these and other forms of cancer, including malignant melanoma and kidney cancer (Rosenberg et al., *N. Engl. J. Med.* 316:889-897 (1987)). LAK cell
10 treatment requires that the patient first receive IL-2, followed by leukapheresis and then an *ex vivo* incubation and culture of the harvested autologous blood cells in the presence of IL-2 for a few days. The LAK cells must be reinfused along with relatively high doses of IL-2 to complete the therapy. This purging treatment is expensive and can cause serious side effects. These include
15 fluid retention, pulmonary edema, drop in blood pressure, and high fever. In some cases in which these side effects occur, intensive care unit management is required.

Purging techniques have been applied in other circumstances as well. Cytotoxic drugs or monoclonal antibodies combined with complement, and
20 toxins, may be administered in order to bring about remission. In such cases bone marrow or blood stem cells, purged to reduce the number of residual leukemic cells present, have been infused back into the patient after the drug treatment (Uckun et al., *Blood* 79:1094 (1992)). Gene marking studies have shown that unpurged bone marrow may contribute to relapse in patients
25 presumed to be in remission (Brenner et al., *Lancet* 341:85 (1993)). This suggests that some form of purging of autologous marrow or blood prior to transplantation is necessary (Klingemann et al., *Biol. Blood Marrow Transplant.* 2:68-69 (1996)).

Recently, preclinical studies have also demonstrated promising antitumor activity *in vivo* with a lethally irradiated, MHC-unrestricted, cytotoxic T-cell leukemic clone (TALL-104) (Cesano et al., *Cancer Immunol. Immunother.* 40:139-151 (1995); Cesano et al., *Blood* 87:393-403 (1996)).

- 5 These cells were derived from leukemia T cell lines obtained from patients having acute T lymphoblastic leukemias (ALL). They bear the CD3 cell surface marker, but not the CD56 marker, in distinction to NK cells which have the converse immunophenotype (CD3⁻ CD56⁺). Adoptive transfer of these cells was able to eliminate human leukemic cell lines in xenografted
- 10 severe combined immunodeficient (SCID) mice and to induce remissions of spontaneous lymphomas in dogs without producing T-cell leukemia in the animal models (Cesano et al. (1995); Cesano et al. (1996); Cesano et al., *J. Clin. Invest.* 94:1076-1084 (1994); Cesano et al., *Cancer Res.* 56:3021-3029 (1996)).
- 15 In spite of the advantageous properties of NK cells in killing tumor cells and virus-infected cells, they remain difficult to work with and to apply in immunotherapy. It is difficult to expand NK cells *ex vivo* that maintain their tumor-targeting, tumoricidal, and viricidal capabilities *in vivo*. This remains a major obstacle to their clinical use in adoptive cell immunotherapy (Melder et
- 20 al., *Cancer Research* 48:3461-3469 (1988); Stephen et al., *Leuk. Lymphoma* 377-399 (1992); Rosenberg et al., *New Engl. J. Med.* 316:889-897 (1987)). Studies of the mechanisms whereby NK cells exert their tumoricidal and viricidal effects are also limited by difficulties in enriching the NK cell fractions without compromising their biological functions and in obtaining pure NK cells
- 25 that are not contaminated by T cells or other immune effector cells. In an attempt to overcome these problems, many investigators have turned to the use of established NK-like cell lines to explore the mechanisms whereby target cells are susceptible to cytotoxic cells (Hercend et al., *Nature* 301:158-

160 (1983); Yodoi et al., *J. Immunol.* 134:1623-1630 (1985); Fernandez et al.,
Blood 67:925-930 (1986); Robertson et al., *Exp. Hematol.* 24:406-415 (1996);
Gong et al., *Leukemia* 8:652-658 (1994)). NK cell lines described in earlier
work carry T lymphocyte-associated surface markers, in spite of the fact that
5 they were developed from precursor cells depleted of T cells (Rosenberg, et
al. (1987); Hercend, et al., (1983)).

There thus remains a need for a method of treating a pathology related
to cancer or a viral infection with a natural killer cell line that maintains
viability and therapeutic effectiveness against a variety of tumor classes.
10 This need encompasses therapeutic methods in which samples from a
mammal are treated *ex vivo* with natural killer cells, as well as methods of
treatment of these pathologies with natural killer cells *in vivo* in a mammal.
There is also a need for a natural killer cell line that maintains its own
propensity for viability and cytolytic activity by secreting a cytokine which
15 fosters these properties. There also remains a need for such natural killer cell
lines which are modified to be more effective, convenient, and/or useful in
treatment of cancer and viral infection. It is the objective of this invention to
provide NK cells and methods that address these needs.

SUMMARY OF THE INVENTION

20 The cell line described by Gong et al. (1994), termed NK-92,
proliferates in the presence of IL-2 and has high cytolytic activity against a
variety of cancers. The present invention employs the NK-92 cell line, as well
as modified NK-92 cell lines, to provide cancer treatment and virus treatment
systems. The invention also provides the vectors that transfect NK-92, as
25 well as the modified NK-92 cells. For purposes of this invention and unless
indicated otherwise, the term "NK-92" is intended to refer to the original NK-
92 cell lines as well as the modified NK-92 cell lines disclosed herein.

One aspect of the invention provides a vector for transfecting NK-92 cells, wherein the vector includes a nucleic acid sequence encoding a protein that is either a cytokine which promotes the growth of the NK-92 cells, a cellular component responsive to an agent, a cancer cell receptor molecule, or any combination of these proteins. When transfected with the vector, the NK-92 cells constitutively express the protein. In an important embodiment, the protein is the cytokine interleukin 2. In especially important embodiments of this aspect of the invention, the vectors are MFG-hIL-2 and pCEP4-LTRhIL-2. In additional significant embodiments, the protein is a cellular component responsive to an agent, such that when the vector transfects NK-92 cells and the agent is taken up by the cells, the cells are inactivated. In still more significant embodiments the agent is either acyclovir or gancyclovir. A further embodiment of the invention provides a cell population containing NK-92 cells that have been modified by a physical treatment or by transfection with a vector.

In significant embodiments of this population, the physical treatment renders them non-proliferative yet does not significantly diminish the cytotoxicity of the cells, and in particularly significant embodiments, the treatment is irradiation. In additional important embodiments the cells have been transfected by a vector that encodes a cytokine promoting the growth of the cells. The cells secrete the cytokine both upon being cultured under conditions that promote cytokine secretion or *in vivo* upon being introduced into a mammal. In particularly important embodiments of this aspect of the invention, the cytokine is interleukin 2. In still further important embodiments, the NK-92 cells are the cells NK-92MI, modified by transfection with the vector MFG-hIL-2 encoding interleukin-2, and the cells NK-92CI modified by transfection with the vector pCEP4-LTRhIL-2 encoding interleukin-2. The NK-92MI and NK-92CI cell lines was have been deposited into the general

depository of the American Type Culture Collection (ATCC), 10801 University
Bldv., Manassas, VA 20110-2209, on September 3, 1998, was assigned
ATCC Deposit No. CRL-2408, was moved to the patent depository on April 4,
2005, and was assigned Deposit No. PTA-6671. The NK-92Cl cell line was
5 deposited into the general depository of ATCC on September 3, 1998, was
assigned ATCC Deposit No. CRL-2409, was moved to the patent depository
on April 4, 2005, and was assigned ATCC Deposit No. PTA-6672. under the
designations CRL-2408 and CRL-2409, respectively.—In additional important

embodiments, the NK-92 cells are transfected by a vector including a
10 sequence that encodes a cellular component responsive to an agent such
that, when the NK-92 cell so transfected takes up the agent, the cell is
inactivated. In particularly important embodiments thereof, the agent is
acyclovir or gancyclovir. In yet additional embodiments, the cell population is
transfected with a vector encoding a cancer cell receptor molecule.

15 The present invention also provides a method of purging cells related
to a pathology from a biological sample including the steps of (i) obtaining a
biological sample from a mammal that is suspected of containing cells related
to the pathology, and (ii) contacting the sample with a medium comprising
NK-92 or modified NK-92 natural killer cells, wherein the modified NK-92 cells
20 have been modified by a physical treatment or by transfection with a vector.
In significant embodiments of this method, the pathology is a cancer, or is an
infection by a pathogenic virus such as human immunodeficiency virus (HIV),
Epstein-Barr virus (EBV), cytomegalovirus (CMV), or herpes virus. In
additional important embodiments, the modified NK-92 cells have undergone
25 a physical treatment that renders them non-proliferative, yet which does not
significantly diminish their cytotoxicity, or have been transfected with a vector,
or they have been treated by any combination of these modifications. In
significant embodiments of this method, the vector encodes a cytokine that

promotes the growth of the cells, a protein that is responsive to an agent, a cancer cell receptor molecule, or a combination of these coding sequences. In a further embodiment, the medium also includes a cytokine that promotes the growth of the cells. The sample, once purged of cancer cells, may be
5 further treated, including, for example, being returned to the mammal from which it was obtained. In important embodiments of the method, the biological sample is blood or bone marrow, the mammal is a human, and/or the natural killer cell is immobilized on a support.

The invention additionally provides a method of treating a pathology
10 *ex vivo* in a mammal including the steps of (i) obtaining a biological sample suspected of containing cells related to the pathology from the mammal; (ii) contacting the biological sample with a medium including natural killer cells, either NK-92 cells or modified NK-92 cells that have been modified by a physical treatment or by transfection with a vector, thereby selectively
15 destroying the cells related to the pathology in the sample and producing a purged sample, and (iii) returning the purged sample to the mammal. The pathology may be a cancer, such as a leukemia, a lymphoma, or a multiple myeloma. Alternatively, the pathology may be infection by a pathogenic virus such as HIV, EBV, CMV, or herpes. In this method the natural killer cells may
20 be NK-92 itself or modified NK-92 cells. Examples of such modified NK-92 cells include those that have been modified by a physical treatment that renders them non-proliferative yet does not significantly diminish their cytotoxicity, and modification by transfection with a vector. The vector encodes a cytokine that promotes the growth of the cells, or a protein that is
25 responsive to an agent, or a cancer cell receptor molecule, or the vector may include any combination of these modifications. In important embodiments of this method, the biological sample is blood or bone marrow, the mammal is a human, and/or the natural killer cell is immobilized on a support. In additional

significant embodiments, the medium further includes a cytokine that promotes the growth of the cells, and/or the cancer is a leukemia, a lymphoma or a multiple myeloma.

The present invention further provides a method of treating a pathology
5 *in vivo* in a mammal including the step of administering to the mammal a medium comprising natural killer cells, either NK-92 cells or NK-92 cells that have been modified by a physical treatment that renders them non-proliferative yet does not significantly diminish their cytotoxicity, by treatment that inhibits expression of HLA antigens on the NK-92 cell surface, or by
10 transfection with a vector. The vector encodes a cytokine that promotes the growth of the cells, or a protein that is responsive to an agent, or a cancer cell receptor molecule, or they have been treated by any combination of these modifications. In important embodiments, the pathology is a cancer, such as a leukemia, a lymphoma, or a multiple myeloma. Alternatively, in important
15 embodiments the pathology is infection by a pathogenic virus such as HIV, EBV, CMV, or herpes. Advantageous embodiments of this method include administering the cells intravenously to a human and administering a cytokine that promotes the growth of the cells to the mammal in conjunction with administering the medium comprising the natural killer cell. The present
20 methods are especially adapted for the treatment of leukemia, lymphoma or multiple myeloma.

In yet an additional embodiment of the *in vivo* method of treating cancer, the NK-92 is modified by transfection with a vector comprising an element responsive to an agent such that when the agent is taken up by the
25 cell, the cell is inactivated. According to this method, an amount of the agent effective to inactivate the cell can be administered to a mammal after a time sufficient for the natural killer cell to treat the cancer has elapsed or at a time desirable to effectively end the treatment. A significant aspect of this

embodiment is one in which the agent is acyclovir or gancyclovir. Such transfected cells can, in effect, be "turned off" as desired by administering the agent.

BRIEF DESCRIPTION OF THE DRAWING

5 Figure 1. Cytotoxic activity of NK-92 against different leukemic target cell lines tested in a 4 hour ^{51}Cr release assay. The results represent the mean \pm the standard deviation (SD) for three replicate experiments.

 Figure 2. Cytotoxicity of NK-92 after IL-2 deprivation. NK-92 cells were cultured in enriched alpha medium (MyelocultTM, StemCell
10 Technologies, Vancouver, BC) without IL-2. Cytotoxicity was measured daily with the ^{51}Cr -release assay against K562-neo' or Daudi target cells. The Figure shows results from one representative experiment at the E:T ratio of 10:1.

 Figure 3. Effect of various doses of γ radiation on the cytolytic potential
15 of NK-92 cells. NK-92 cells were irradiated with a ^{137}Cs source using doses ranging from 200 to 1000 cGy. To allow for recovery, cells were left in medium containing IL-2 for 24 hours before cytotoxicity was measured in a 4 hour ^{51}Cr release assay against the target cell line K562.

 Figure 4. Survival curves of NK-92 cells after γ -irradiation. NK-92 cells
20 were irradiated with a γ ray source at doses of 300, 500, 1000, and 3000 cGy. Viability of NK-92 cells was determined by trypan blue staining. The maximal achievable concentration of the non-irradiated NK-92 cells in culture was about $1.5 \times 10^6/\text{mL}$. The cells had to be fed to prevent overgrowth.

 Figure 5. Effect of γ -irradiation on the *in vitro* colony formation of NK-
25 92 cells. NK-92 cells were cultured in agar-based medium supplemented with recombinant human IL-2 (rhIL-2).

Figure 6. Effect of various radiation doses on the cytolytic potential of NK-92 cells. NK-92 cells were γ -irradiated at doses of 300, 500, 1000, and 3000 cGy. ^{51}Cr -labeled leukemic target cells K562 (Panel A) and HL60 (Panel B) as well as two patient-derived leukemic samples TA27 (Panel C) and BA25 (Panel D) were tested for susceptibility to cytotoxicity by irradiated and non-irradiated (NR) NK-92 cells. The results of 4 hr chromium release assays are expressed as 30% lytic units/ 10^8 effector cells.

Figure 7. Selective killing of patient-derived leukemic cells by NK-92 cells. ^{51}Cr -labeled leukemic target cells derived from 40 patients [9 acute myeloid leukemia (AML) cases, 11 chronic myeloid leukemia (CML) cases, 14 B-lineage-acute lymphoblastic leukemia (ALL) cases and 6 T-ALL cases] and T cell depleted normal bone marrow cells from 14 normal donors were tested for susceptibility to cytotoxicity by NK-92 cells at four different E:T ratios. The results of a 4 hr chromium release assay are expressed as 30% lytic units/ 10^8 effector cells.

Figure 8. *In vitro* (Panels A and B) and *in vivo* (Panels C and D) antileukemic efficacy of NK-92 cells against K562 and HL60 leukemias as compared to human LAK cells and other effectors. ^{51}Cr labeled K562 (Panel A) and HL60 (Panel B) cells were tested for susceptibility to cytotoxicity by NK-92 cells in comparison with various known effector cells [LAK, NK ($\text{CD}3^+ \text{CD}56^+$), and T cells ($\text{CD}3^+ \text{CD}56^+$)] at indicated E:T ratios in a 4 hr CRA assay. Results are means \pm SD of three separate tests for NK-92 cells, and two tests of different donor-derived effectors for LAK, $\text{CD}56^+$ and $\text{CD}3^+$ cells. SCID mice were inoculated subcutaneously with K562 cells (Panel C) or HL60 cells (Panel D) (5×10^8 cells per mouse) alone or in combination with NK-92, LAK, or NK cells at a 4:1 E:T ratio. As a measure of the tumor sizes, their surface areas were measured once a week post inoculation (n=5).

Figure 9. Antileukemic effect of NK-92 cells, allogeneic cytotoxic T lymphocyte (CTL) cells and other effector cells against a patient-derived acute T lymphoblastic leukemia (T-ALL) determined *in vitro* and *in vivo*.

Panel A: *In vitro* specific killing of T-ALL (TA27) target cells by NK-92, CTL, and other effector cells, was determined by a 4 hr ^{51}Cr -release assay using the indicated E:T ratios. Results are means \pm SD of two or three separate tests. Panel B: SCID mice were inoculated subcutaneously with TA27 cells (5×10^6 each mouse) alone or co-inoculated with NK-92, CTL or other effector cells at a 4:1 E:T ratio. Recombinant human IL-2 (rhIL-2) was administered to the mice intraperitoneally for two weeks at the dose of 5×10^4 U every other day. Leukemic tumor areas were measured once a week post inoculation (n=5).

Figure 10. Survival of SCID mice bearing T-ALL (TA27) leukemia co-inoculated with NK-92 cells as compared with co-inoculation with allogeneic CTL or irradiated TALL-104 cells.

Figure 11. Survival of SCID mice bearing T-ALL (TA27) after treatment with NK-92 cells. Mice received 5×10^6 TA27 cells intraperitoneally (I.P.). NK-92 cells (2×10^7) were injected I.P. once, or 5 times (on days 1, 3, 5, 7 and 9), with or without the addition of rhIL-2 every other day for two weeks.

Figure 12. Survival of SCID mice bearing pre-B-ALL (BA31) after treatment with NK-92 cells. Mice received 5×10^6 BA31 cells I.P. NK-92 (2×10^7) cells were injected I.P. for a total of 5 doses, on days 1, 3, 5, 7 and 9. Mice in the indicated groups received rhIL-2 every other day for two weeks.

Figure 13. Survival of SCID mice bearing human AML (MA26) after treatment with NK-92 cells. Mice received 5×10^6 MA26 leukemia cells I.P. NK-92 (2×10^7) cells were injected I.P. on days 1, 3, 5, 7 and 9 for a total of five doses. Mice in the indicated groups received rhIL-2 every other day for two weeks.

Figure 14. Diagrammatic map of plasmid MFG-hIL-2.

Figure 15. Diagrammatic map of plasmid pCEP4-LTR.hIL-2.

Figure 16. Cytotoxicity of NK-92, NK-92MI and NK-92CI against K562 and Raji target cells. The cytotoxic activities of the IL-2 transfectants were compared to that of the parental cell line. NK cells were mixed with ⁵¹Cr-labeled K562 (Panel A) or Raji (Panel B) cells at effector:target ratios of 1:1, 5:1, 10:1 and 20:1 for a 4 hour chromium release assay. The cytotoxicities of NK-92 (•), NK-92MI (▲) and NK-92CI (■) are shown.

Figure 17. Effect of NK-92 MI and NK-92CI on hematopoietic progenitors. To assay the effect of the NK-92 cells on normal hematopoietic progenitors, a clonogenic assay was performed. Normal PBMCs were incubated with irradiated NK-92MI or NK-92CI at various NK:PBMC ratios ranging from 1:1 to 1:1000 for 48 hours. The cells were plated in methylcellulose at concentrations to give 10-100 colonies per dish after 14 days. Clonogenic output of PBMCs incubated with NK-92MI (white bars) and NK-92CI (gray bars) is expressed as either total number of colonies or subclassified on the basis of colony type (BFU-E, CFU-GM and CFU-GEMM).

Figure 18. Effect of irradiation on NK-92, NK-92MI and NK-92CI proliferation and viability. To assess the effect of irradiation on the parental and transfected NK-92 cells, cells were exposed to 0, 500, 1,000, 1,500, and 2,000 cGy doses of radiation and assayed for proliferation by a standard ³H-thymidine incorporation assay. Panel A: Proliferation of NK-92 (•), NK-92MI (▲) and NK-92CI (■) is expressed as a percentage of control (unirradiated cells). Panel B: Cells were exposed to 0, 250, 500, 1,000, and 2,000 cGy of irradiation and assessed by trypan blue exclusion for viability after 24 (black bars), 48 (gray bars) and 72 hours (white bars).

Figure 19. Effect of irradiation on NK-92, NK-92MI and NK-92CI cytotoxicity. To assess the effect of irradiation on cytotoxicity of the NK cells,

NK-92, NK-92MI and NK-92CI were irradiated at 0, 1,000, and 2,000 cGy and tested after three days for cytotoxicity against K562 (Panel A) and Raji (Panel B) cells.

DETAILED DESCRIPTION OF THE INVENTION

5 The present invention relates to methods of treating a biological sample or a mammal suspected of having a pathology such as a cancer or an infection by a virus. Certain natural killer cells which are cytolytic for the cells affected by the pathology are employed. The treatment results in significant diminution of the number, or, in some cases, the elimination, of malignant or
10 cancerous cells, or virus-infected cells, in the sample or mammal. The natural killer cells of this invention are designated NK-92 cells and include certain treated or transfected modifications of NK-92 cells. These cells are highly effective in purging cancer cells *ex vivo* and in destroying cancer cells *in vivo*.

15 As used in the present invention, "cytotoxic T lymphocytes" (CTL) relate to immune cells which kill antigen-specific target cells. CTL are MHC class I-restricted. As used in the present invention, lymphokine activated killer (LAK) cells relate to cells of the immune system that have antitumor killing activity. They are obtained from a population of cells, such as
20 peripheral blood mononuclear cells, upon activation by treatment with lymphokines. LAK cells have essentially no effect on normal cells.

 As used to describe the present invention, "natural killer (NK) cells" are cells of the immune system that kill target cells in the absence of a specific antigenic stimulus, and without restriction according to MHC class.
25 Target cells may be tumor cells or cells harboring viruses. NK cells are characterized by the presence of CD56 and the absence of CD3 surface markers. The present invention is based on an immortal NK cell line, NK-92, originally obtained from a patient having non-Hodgkin's lymphoma. As used

to describe the present invention, a modified NK-92 cell is an NK-92 cell which has been further treated to endow it with properties not found in the parent from which it is derived. Such treatments include, for example, physical treatments, chemical and/or biological treatments, and the like. The
5 treatments confer properties upon the modified NK-92 cells that render them more advantageous for the purposes of the invention.

As used to describe the present invention, the terms "cytotoxic" and "cytolytic", when used to describe the activity of effector cells such as NK cells, are intended to be synonymous. In general, cytotoxic activity relates to
10 killing of target cells by any of a variety of biological, biochemical, or biophysical mechanisms. Cytolysis refers more specifically to activity in which the effector lyses the plasma membrane of the target cell, thereby destroying its physical integrity. This results in the killing of the target cell. Without wishing to be bound by theory, it is believed that the cytotoxic effect
15 of NK cells is due to cytolysis.

As used to describe the present invention, "target cells" are the cells that are killed by the cytotoxic activity of the NK cells of the invention. These include in particular cells that are malignant or otherwise derived from a cancer, and cells that are infected by pathogenic viruses such as HIV, EBV,
20 CMV, or herpes.

As used to describe the present invention, "purging" relates to killing of target cells by effector cells such as NK cells *ex vivo*. The target cells may be included in a biological sample obtained from a mammal believed to be suffering from a pathology related to the presence of the target cell in the
25 sample. The pathology may be a cancer or malignancy due to tumor cells in the sample, and may be treated by purging the sample of the tumor cells and returning the sample to the body of the mammal.

As used to describe the present invention, "inactivation" of the NK-92 cells renders them incapable of growth and/or their normal function, in particular, their cytotoxic activity. Inactivation may also relate to the death of the NK-92 cells. It is envisioned that the NK-92 cells may be inactivated after
5 they have effectively purged an *ex vivo* sample of cells related to a pathology in a therapeutic application, or after they have resided within the body of a mammal a sufficient period of time to effectively kill many or all target cells residing within the body. Inactivation may be induced, by way of nonlimiting example, by administering an inactivating agent to which the NK-92 cells are
10 sensitive.

As used herein, a "vector" relates to a nucleic acid which functions to incorporate a particular nucleic acid segment, such as a sequence encoding a particular gene, into a cell. In most cases, the cell does not naturally contain the gene, so that the particular gene being incorporated is a heterologous
15 gene. A vector may include additional functional elements that direct and/or regulate transcription of the inserted gene or fragment. The regulatory sequence is operably positioned with respect to the protein-encoding sequence such that, when the vector is introduced into a suitable host cell and the regulatory sequence exerts its effect, the protein is expressed.
20 Regulatory sequences may include, by way of non-limiting example, a promoter, regions upstream or downstream of the promoter such as enhancers that may regulate the transcriptional activity of the promoter, and an origin of replication. A vector may additionally include appropriate restriction sites, antibiotic resistance or other markers for selection of vector
25 containing cells, RNA splice junctions, a transcription termination region, and so forth.

As used to describe the present invention, "cancer", "tumor", and "malignancy" all relate equivalently to a hyperplasia of a tissue or organ. If

the tissue is a part of the lymphatic or immune system, malignant cells may include non-solid tumors of circulating cells. Malignancies of other tissues or organs may produce solid tumors. In general, the methods of the present invention may be used in the treatment of lymphatic cells, circulating immune
5 cells, and solid tumors.

As used to describe the present invention, a "pathogenic virus" is a virus causing disease in a host. The pathogenic virus infects cells of the host animal and the consequence of such infection is a deterioration in the health of the host. Pathogenic viruses envisioned by the present invention include,
10 but are not limited to, HIV, EBV, CMV, and herpes.

Natural Killer Cell NK-92. The NK-92 cell line has been described by Gong et al. (1994). It is found to exhibit the CD56^{bright}, CD2, CD7, CD11a, CD28, CD45, and CD54 surface markers. It furthermore does not display the CD1, CD3, CD4, CD5, CD8, CD10, CD14, CD16, CD19, CD20, CD23, and
15 CD34 markers. Growth of NK-92 cells in culture is dependent upon the presence of recombinant interleukin 2 (rIL-2), with a dose as low as 10 IU/mL being sufficient to maintain proliferation. IL-7 and IL-12 do not support long-term growth, nor do other cytokines tested, including IL-1 α , IL-6, tumor necrosis factor α , interferon α , and interferon γ . NK-92 is highly effective in
20 killing certain tumor cells, such as K562 (erythroleukemia) and Daudi (Burkitt lymphoma) cells, for it has high cytotoxicity even at a low effector:target (E:T) ratio of 1:1 (Gong et al. (1994)). In addition, NK-92 cells have high cytotoxic activity against 8E5 cells, which are infected with HIV and produce HIV
viruses. NK-92 cells were deposited into the general depository of with
25 the American Type Culture Collection (ATCC), 10801 University Blvd.,
Masassas, VA 20110-2209, on September 3, 1998, was assigned ATCC
Deposit No. ~~7~~-designation-CRL-2407, was moved to the patent depository on
April 4, 2005, and was assigned ATCC Deposit No. PTA-6670.

NK-92 cells are readily maintained in culture medium, such as enriched alpha minimum essential medium (MEM; Sigma Chemical Co., St. Louis, MO) supplemented with fetal calf serum (for example, at 12.5%; Sigma Chemical Co., St. Louis, MO), and horse serum (for example, at 12.5%;
5 Sigma Chemical Co., St. Louis, MO). Initially, 10^6 M hydrocortisone is required, but in subsequent passages it is found that hydrocortisone may be omitted. In addition, IL-2, such as recombinant human IL-2 (500 U/mL; Chiron, Emeryville, CA), is required for long-term growth. When suspension cultures are maintained in this fashion with semiweekly changes of medium,
10 the cells exhibit a doubling time of about 24 h.

NK-92 cells *in vitro* demonstrate lytic activity against a broad range of malignant target cells. These include cell lines derived from circulating target cells such as acute and chronic lymphoblastic and myelogenous leukemia, lymphoma, myeloma, melanoma, as well as cells from solid tumors such as
15 prostate cancer, neuroblastoma, and breast cancer cell lines. This effect is observed even at very low effector:target ratios. This lysis is superior to cytotoxicity obtained from normal peripheral blood mononuclear cells stimulated for four days with IL-2.

Vector for transfecting mammalian cells to produce cytokine. The
20 present invention provides NK-92 cells which have been modified by transfection with a vector that directs the secretion of a cytokine, such as IL-2. In order that NK-92 cells maintain long-term growth and cytolytic function, they generally must be supplied with IL-2. A vector encoding the gene for human IL-2, and which also contains a control element directing the synthesis
25 of the IL-2 gene product is therefore of great utility in the invention. NK-92 cells bearing such a vector secrete the IL-2 needed for cytolytic activity in a therapeutic setting; thus, IL-2 from an exogenous source is not required. The control element is one which directs the synthesis of IL-2 as a constitutive

product, i.e., one that is not dependent upon induction. Methods for constructing and employing vectors are described in general terms in "Current Protocols in Molecular Biology", Ausubel et al., John Wiley and Sons, New York (1987, updated quarterly), and "Molecular Cloning: A Laboratory Manual
5 2nd Ed.", Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989), which are incorporated herein by reference.

Modified NK-92 transfected to produce cytokine, and method of transfecting. Modified NK-92 cells that secrete a cytokine may be prepared by inserting a vector that directs the synthesis and secretion of the cytokine
10 into the cells. In important aspects of the invention, the cytokine is IL-2. Methods of introducing a vector into a mammalian cell are well known to workers of ordinary skill in molecular biology and cellular immunology, and are described in Ausubel et al. (1987, updated quarterly) and Sambrook et al. (1989). The vectors encoding the cytokine encompass as well control
15 elements that lead to constitutive synthesis of the cytokine when incorporated into the NK-92 cells.

When cultured under appropriate conditions that promote cytokine secretion the transfected NK-92 cells secrete IL-2 or other cytokine. Since the vector directs constitutive synthesis of the cytokine, nutrient cultures in
20 which the NK-92 cells are known to grow and to exhibit their normal cytolytic function are sufficient for the transfected cells to secrete the cytokine. For the same reason, the transfected cells secrete the cytokine *in vivo* when they are introduced within the body of a mammal.

NK-92 cells transfected with a vector that directs secretion of a
25 cytokine such as IL-2 are useful in the *ex vivo* treatment of a biological sample drawn from a mammal which is suspected of containing malignant cells. By treating the malignant cells with these modified NK-92 cells, the need for applying exogenous IL-2 or other cytokine is obviated. These

modified NK-92 cells are useful, for the same reasons, in the *in vivo* treatment of a mammal suffering from a malignancy. The modified NK-92 cells exert their cytolytic effect against the malignant cells when introduced into the body of the mammal. Examples of such cells in the present invention are
5 designated NK-92MI and NK-92CI.

NK-92 cells that are cytolytic but not capable of proliferation. An additional modified NK-92 cell of the invention is one that has been treated in such a way that it is no longer able to proliferate, yet whose cytotoxic activity is preserved. One way of achieving this state is by γ irradiation. Additional
10 forms of radiation, including, for example, ultraviolet radiation, may be employed. Suitable sources to use for this purpose include, for example, a ^{137}Cs source (Cis-US, Bedford, MA; Gammacell 40, Atomic Energy of Canada Ltd., Canada). Additionally, proliferative activity may be abrogated by treatment with chemical agents which inhibit DNA synthesis. An example of
15 such an agent is mitomycin C.

Vector for transfecting NK-92 with an element responsive to an inactivating agent. The NK-92 cells may also be modified by transfection with a vector such that, when the cell takes up a specific agent, the cell is inactivated. The vector includes a sequence that encodes a cellular
20 component responsive to the agent, such that when the vector transfects a cell and the agent is taken up by the cell, the cell is inactivated. In preferred embodiments, the agent is acyclovir or gancyclovir. The vector also contains a control element directing the synthesis of the cellular component as a constitutive product.

25 The NK-92 cell transfected with the vector described in the preceding paragraph maintains its characteristic growth and cytolytic activity in the absence of the agent. At a point in time, for example, when an *ex vivo* sample has been purged of malignant cells by the action of the NK-92 cells,

or when the NK-92 cells administered *in vivo* have effectively exerted their cytolytic activity within a mammalian body, or when it desired that the treatment be stopped for any reason, the agent may be administered. The agent interacts with the cellular component sensitive to the agent encoded in
5 the vector. The interaction of the agent with the cellular component induces the inactivation of the NK-92 cells. Inactivation may range from loss of characteristic cytolytic function to death of the cells.

This property of the modified NK-92 cells is significant because the parent NK-92 cells are derived from a tumor cell line that may continue
10 propagating in a sample reintroduced into a mammal after *ex vivo* therapy, or *in vivo* when so administered. It is therefore important to ablate the cells after they have carried out their therapeutic function. Rendering the cells sensitive to an agent such as acyclovir or gancyclovir is an advantageous way of achieving this objective.

15 Vector for transfecting NK-92 with an altered HLA cell surface molecule. The HLA cell surface protein, involved in presenting antigens to other cells of the immune system, includes a non-immunospecific subunit, the protein β_2 -microglobulin. If this protein is altered or mutated, the HLA protein loses its affinity for the T-cell receptor to which it ordinarily binds. The β_2 -
20 microglobulin gene in NK-92 cells of the invention may be mutated by site specific mutagenesis in order to transform its properties in this way. The result is an NK-92 cell which no longer has a high affinity for T-cell receptors. As a result, the NK-92 cell modified in this way remains within the host organism for a longer period of time, rather than being eliminated by the
25 action of the host's cellular immune response.

Vector for transfecting NK-92 with a gene encoding a cancer cell receptor molecule. The NK-92 cells may also be modified by transfection with a vector such that the cells constitutively express a receptor for a cancer

cell. Cancer cells express cell surface molecules that are idiosyncratic for the origin of the cancer, and frequently are also idiosyncratic for the individual host. The CTL population in such diseased patients may have been activated by exposure to the cells of the growing cancer. Such activated CTL
5 express cell surface proteins that are specific for, or target, the cells of the cancer. These CTL may be isolated, the gene for the targeting receptor identified, isolated, and transfected into the NK-92 cells of the invention. This confers on the NK-92 cells the capability of likewise specifically targeting the cancer cells present in the individual host. This has the effect of enhancing
10 the specificity of the cytotoxic activity of the NK-92 cells toward the cancer cells of that individual. The corresponding process would be carried out for each host suffering from cancer, taking advantage of the idiosyncratic specificity of the CTL targeting moiety in each case.

Methods of treating. The natural killer cells of the invention are
15 employed in methods of treating biological samples in order to purge them of cells from a cancer, a malignancy, or a tumor, or cells infected by a pathogenic virus. The NK cells include by way of nonlimiting example, NK-92, and modified NK-92 cells, such as NK-92MI and NK-92CI, as well as other modified NK-92 cells envisioned within the scope of this invention. The
20 NK-92MI and NK-92CI cells are modified by transfection with vectors that result in the secretion of IL-2. In addition, any of the NK-92, NK-92MI, and NK-92CI cells may be treated such that they maintain the cytolytic activity of the untreated cells but cannot proliferate. The NK cells so treated may also be equivalent cell lines which have the properties such as cytotoxicity and
25 NK-specific cell surface markers described herein. Malignancies of the immune system, the lymphatic system, and the hematopoietic system may be treated by the methods of the invention. In addition, formed tumors and solid

tumors may also be treated. Infections by pathogenic viruses, such as HIV, EBV, CMV, and herpes may also be treated.

Treating a biological sample. *In vitro* biological samples may be treated experimentally or therapeutically in order to eliminate malignant cells, or virus-infected cells, in an effective manner. The sample may be drawn from a mammal and maintained *in vitro* in an appropriate culture medium. Such media are well known to workers of skill in cell biology, cellular immunology, and oncology. Media and cell culture techniques are presented in general terms in, for example, Freshney, R.I., "Culture of Animal Cells, 3rd Ed.", Wiley-Liss, New York (1994), and in Martin, B. M., "Tissue Culture Techniques, An Introduction", Birkhauser, Boston, MA (1994), which are incorporated herein by reference. The biological sample is established in culture *in vitro*, and contacted with a medium that includes the natural killer cells of the present invention. The cytolytic activity of the NK cells effectively eliminates the malignant cells or the virus-infected cells from the sample. The prevalence and depletion of the target cells may be traced by any of a number of methods well known to those of skill in the fields of cell biology and cellular immunology. These include indirect immunofluorescence microscopy to assay for intact tumor cells or virus-bearing cells, fluorescent-activated cell sorting, chromium release assays, and the like.

Treating a cancer or virus infection ex vivo: purging. The present invention additionally encompasses the *ex vivo* treatment of a biological sample suspected of containing cancer cells or virus-infected cells by contacting the sample with the NK cells of the invention. The biological sample is drawn from the body of a mammal, such as a human, and may be blood, bone marrow cells, or similar tissues or cells from an organ afflicted with a cancer. Methods for obtaining such samples are well known to workers in the fields of cellular immunology, oncology, and surgery. They

include sampling blood in well known ways, or obtaining biopsies from the bone marrow or other tissue or organ. The cancer cells or virus-infected cells contained in the sample are effectively eliminated due to the cytotoxic activity of the NK-92 cells. The sample may then be returned to the body of the mammal from which it was obtained.

5 The NK-92 cells used to treat the sample may be freely suspended in the medium. It is generally preferred that the purged sample, prior to being returned to the body of the mammal from which it was obtained, be rid of NK-92 cells that may continue growing, since they arose originally from a proliferating lymphoma. The invention envisions several ways of accomplishing this objective. In one embodiment, the NK cells, prior to use, are irradiated with γ rays or with ultraviolet light to the extent that they maintain their cytolytic activity but are not capable of growth. In an additional embodiment, the NK cells are permanently immobilized on a macroscopic solid support. The support with the NK cells attached may then be physically separated from the cells of the biological sample, for example by centrifugation, or filtration with a column which permits the unbound cells of the sample to pass through, or like technique. Suitable solid supports include particles of polyacrylamide, agarose, cellulose, Sepharose™ (Pharmacia, Piscataway, NJ), celite, and the like, and may be supplied with groups such as an aldehyde, carbonyldiimidazole, broamoacetyl, epichlorhydrin, and the like, which are activated for reaction with cell surface groups. The activated groups on the support react with groups such as amino or carboxyl groups, for example, on the cell surface, thereby immobilizing the cells on the support.

25 In yet a further embodiment, the NK cells may be modified with a vector directing the synthesis of a cellular component sensitive to an agent, such that when the agent is administered to the *ex vivo* sample, the NK-92

cells are inactivated. Examples of such agents include acyclovir or gancyclovir, by way of nonlimiting example. Functionally equivalent vectors, directing the synthesis of alternative cellular components sensitive to different agents, are also envisioned within the scope of this embodiment.

- 5 The NK cells to be used in the methods of the invention may require a cytokine such as IL-2 to maintain their functional effectiveness as cytolytic cells. The cytokine may simply be added to the *ex vivo* preparation. Alternatively, if desired, a modified NK-92 cell bearing a vector directing the constitutive synthesis of the cytokine may be employed. In this way the
10 necessity of furnishing exogenous cytokine is avoided.

Treating a cancer or virus infection *in vivo*: administering NK-92.

- A further method of the invention is directed toward treatment of a cancer or a virus infection *in vivo* in a mammal using NK-92 cells. The cells are administered in a variety of ways. By way of nonlimiting example, the cells
15 may be delivered intravenously, or into a body cavity adjacent to the location of a solid tumor, such as the intraperitoneal cavity, or injected directly within or adjacent to a solid tumor. Intravenous administration, for example, is advantageous in the treatment of leukemias, lymphomas, and comparable malignancies of the lymphatic system, as well as in the treatment of viral
20 infections.

- As has been described in detail in the preceding section, it is desirable to employ methods that eliminate or ablate the NK-92 cells after they have effectively lysed (or otherwise destroyed) the target cells. Certain methods described above may be employed for this purpose, namely, use of irradiated
25 NK-92 cells, and use of NK-92 cells harboring a vector directing the synthesis of a cellular component sensitive to an agent, such that when the agent is administered, the NK-92 cells are inactivated, and equivalent methods. When the cells produce such a component sensitive to the specific agent,

administration of the agent to the mammal is effective to inactivate the NK-92 cells within the mammal.

The NK-92 cells may be administered in conjunction with a cytokine such as IL-2 in order to maintain the functional effectiveness of the cells as cytotoxic effectors. As used to describe the invention, the term "in conjunction" indicates that the cytokine may be administered shortly prior to administration of the NK-92 cells, or it may be given simultaneously with the cells, or shortly after the cells have been administered. The cytokine may also be given at two such times, or at all three times with respect to the time of administering the NK-92 cells. Alternatively, NK-92 cells harboring a vector directing the constitutive synthesis of the cytokine may be employed in the *in vivo* method of treating a cancer. This effectively eliminates the need to furnish exogenous cytokine.

The following examples are included to illustrate the invention and not to limit the invention. All publications or references cited in the present specification are hereby incorporated by reference.

EXAMPLES

Example 1. NK-92 Cells. NK-92 cells (Gong et al. (1994)) were derived from cells obtained from a patient suffering from non-Hodgkin's lymphoma. PBMC from the patient were cultured in enriched alpha MEM supplemented with fetal calf serum (12.5%) and horse serum (12.5%) plus 10^{-6} M hydrocortisone and 1000 U/mL of recombinant human IL-2 (rhIL-2). Cells were cultured at 37°C in humidified air containing 5% CO₂. Subcultures were made after 4 weeks, and propagated indefinitely with twice-weekly changes in medium. In these later stages the hydrocortisone could be omitted without any effect on cell growth. This culture has been designated NK-92 and has been deposited with the American Type Culture Collection (ATCC; Rockville, MD) under designation CLR-2407.

The cells have the morphology of large granular lymphocytes. The cells bear the CD56^{bright}, CD2, CD7, CD11a, CD28, CD45, and CD54 surface markers. In contrast, they do not display the CD1, CD3, CD4, CD5, CD8, CD10, CD14, CD16, CD19, CD20, CD23, and CD34 markers. Growth of NK-92 cells in culture is dependent upon the presence of recombinant interleukin 2 (IL-2), with a dose as low as 10 IU/mL being sufficient to maintain proliferation. IL-7 and IL-12 do not support long-term growth, nor do other cytokines tested, IL-1 α , IL-6, tumor necrosis factor α , interferon α , and interferon γ .

Example 2. Cytotoxic Activity of NK-92 against Different Leukemic

Cell Lines. The cytotoxic activity of NK-92 against K562, Daudi, TF-1, AML-193, and SR-91 cells was determined (Gong et al. (1994)). K562 (erythroleukemia) and Daudi (Burkitt) lymphoma cell lines were obtained from ATCC. They were maintained in continuous suspension culture in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). TF-1 is a myelomonocytic cell line (Kitamura et al., *J. Cell Physiol.* 140:323-334 (1989)) that requires the presence of medium containing 2 ng/mL of human GM-CSF. AML-193 is a myeloid cell line that is maintained in the presence of 10% 5637-conditioned medium (Lange et al., *Blood* 70:192-199 (1987)). Both TF-1 and AML-193 cells were obtained from Dr. D. Hogge, Terry Fox Laboratory, University of British Columbia, Vancouver, BC. SR-91 is a cell line with features of early progenitor cells established by Gong et al. (1994) from a patient with acute lymphoblastic leukemia (ALL) (Klingemann et al., *Leuk. Lymphoma*, 12, 463-470 (1994). It is resistant to both NK and activated-NK (A-NK) cell cytotoxicity. SR-91 is also maintained in RPMI 1640/10% FCS. This cell line can be rendered sensitive to killing by NK-92 by treatment with cytokine. Naki et al., "Induction of sensitivity to the NK-mediated cytotoxicity by TNF- α treatment: Possible role of ICAM-3 and CD44," *Leukemia*, in press.

The cytotoxic activity of NK-92 (effector) against these target cells was assessed by means of a ^{51}Cr release assay (Gong et al. (1994)) using the procedure described by Klingemann et al. (*Cancer Immunol. Immunother.* 33:395-397 (1991)). The percentage of specific cytotoxicity in triplicate specimens was calculated as:

$$\% \text{ } ^{51}\text{Cr release} = \frac{(\text{average experimental cpm} - \text{average spontaneous cpm})}{(\text{average maximum cpm} - \text{average spontaneous cpm})} \times 100$$

Figure 1 presents the results of this determination. It is seen that NK-92 cells kill K562 and Daudi cells with high efficiency. Even at the low E:T ratio of 1:1, 83% of K562 cells and 76% of Daudi cells were killed by NK-92 cells. Susceptibility to killing by NK-92 cells was lower for TF-1 cells (23% at E:T = 1:1) and for AML-193 cells (6% at E:T=1:1). SR-91 cells appear to be resistant to the cytotoxic effect of the NK-92 cells. Without wishing to be bound by theory, it is believed that SR-91 cells lack adhesion molecules necessary to mediate initial binding with NK-92 cells.

Example 3. Cytotoxicity of NK-92 against Leukemia, Lymphoma, and Myeloma Target Cell Lines. K562 (Ph-chromosome positive [Ph⁺] erythroleukemia), HL60 (promyelocytic), U937 (myelomonocytic), KG1 a (variant subline of the AML cell line KG1), DHL-10 (B-cell lymphoma), Daudi (Burkitt's lymphoma), Raji (B-cell lymphoma), Jurkat (T-cell lymphoma), U266 (IgE myeloma), NCI H929 (IgA myeloma), and RPMI 8226 (myeloma, light chain secreting) cell lines were obtained from ATCC. The lymphoma-derived cell lines Ly3 (B-lineage, diffuse large cell), Ly8 (immunoblastic), and Ly13.2 (T-lineage, diffuse large cell) were provided by Dr. H. Messner, Toronto, Ontario. Their characteristics have been described (Chang et al., *Leuk. Lymphoma* 19:165 (1995)). All lines were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM

nonessential amino acids, 50 U/mL penicillin, 25 mM HEPES (StemCell Technologies), and 5% heat-inactive FCS (RPMI/5% FCS) at 37° C in a humidified atmosphere of 5% CO₂ in air.

Cell lysis was determined by a 4-hour ⁵¹Cr release assay using various
5 E:T ratios. To allow for comparison, PBMCs from normal donors were
activated with IL-2 (500 U/mL) for 4 days and ⁵¹Cr release measured against
the same target cells concurrently. The mean of two separate experiments is
presented.

Results of NK-92-mediated cytotoxicity (⁵¹Cr release assay) against
10 various leukemia, lymphoma, and myeloma target cell lines are summarized
in Table 1. For comparison, lysis of the same tumor target cells was also
tested in the same experiment with PBMCs obtained from normal donors.
Those cells had been activated by IL-2 (500 U/mL) for 4 days prior to testing.
Results show that NK-92 cells very effectively lyse all target cells tested.
15 High cytotoxicity is observed even at the low E:T ratio of 1:1. The cytotoxicity
achieved with these cells is significantly higher than that observed with
normal (allogeneic) PBMCs activated under optimal conditions with IL-2 for all
the target cells except RPMI 8226 and U266.

Table 1. Cytotoxic activity of NK-92 cells against various leukemia, lymphoma, and myeloma cell lines

Target		50:1	20:1	10:1	5:1	1:1
HL-60	NK-92	97	90	77	46	40
	PBMCs + IL-2	31	26	17	2	0
K562	NK-92	68	68	64	59	50
	PBMCs + IL-2	63	73	67	51	19
KG1a	NK-92	90	91	80	67	39
	PBMCs + IL-2	15	11	12	6	0
U937	NK-92	99	98	96	91	85
	PBMCs + IL-2	57	43	23	13	2
DHL-10	NK-92	95	95	92	94	80
	PBMCs + IL-2	60	40	24	19	5
Daudi	NK-92	94	87	71	48	39
	PBMCs + IL-2	65	57	29	16	6
Jurkat	NK-92	100	100	98	93	80
	PBMCs + IL-2	67	50	36	27	4
Ly3	NK-92	63	59	53	42	28
	PBMCs + IL-2	47	35	18	6	095
Ly8	NK-92	95	104	102	88	42
	PBMCs + IL-2	67	65	62	59	44
Ly 13.2	NK-92	104	105	100	97	67
	PBMCs + IL-2	61	63	52	4	13
Raji	NK-92	81	75	74	70	54
	PBMCs + IL-2	32	67	57	35	13
NCI H929	NK-92	94	89	89	86	51
	PBMCs + IL-2	75	58	39	24	5
RPMI 8224	NK-92	82	72	70	72	41
	PBMCs + IL-2	95	83	81	67	25

U.S. Patent Appn. Serial No. 10/008,955
Substitute Specification (Marked Up) Dated October 28, 2010
Submitted In Conjunction with
Statement of Hans Klingemann, M.D., Ph.D. Regarding Biological Deposits In
Accordance With 37 C.F.R. §§ 1.801-1.809

U266	NK-92	84	77	85	81	53
	PBMCs + IL-2	84	74	73	56	21

Example 4. Effect of Deprivation of IL-2 on Cytotoxic Activity of NK-92. To test how long NK-92 cells would maintain their cytolytic activity without IL-2 present in the culture medium, NK-92 cells were deprived of IL-2 and ⁵¹Cr-release was measured in 24-hour intervals. Results, summarized in Figure 2, suggest that the cells maintain full cytotoxic activity for at least 48 hours. Thereafter, the activity drops precipitously to negligible levels. Thus, for short-term purging, IL-2 does not have to be present in the cultures to achieve a suitable effect.

Example 5. Co-culture of K562-neo' Cells with PBMC'S and NK-92. The transfection of the K562 cells with the neomycin-resistance (neo') gene has been described (Wong et al., *Bone Marrow Transplant* 18:63 (1966)). Briefly, 5×10^7 K562 cells were suspended in 0.8 mL RPMI 1640/5% FCS and incubated on ice for 10 minutes with 30 µg of the pMCI-Neo plasmid (provided by Dr. K. Humphries, Terry Fox Laboratory, Vancouver, BC). The cells were then exposed to a single voltage pulse (125 µF/0.4kV) at room temperature, allowed to remain in buffer for 10 minutes, transferred into 25-cm² tissue culture flasks (Falcon, Lincoln Park, NJ), and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 2 days. Transfected cells were selected in 0.8% Iscove's methylcellulose medium (StemCell Technologies) supplemented with 30% FCS, 10^{-4} M 2-mercaptoethanol, and 2 mM glutamine, containing 0.8 mg/mL G418 (neomycin) (Gibco-BRL, Grand Island, NY). Neo' clones of K562 cells were identified after 2 weeks, plucked, and maintained in RPMI/10% FCS containing 0.8 mg/mL neomycin. K562-neo' cells cultured for 2 days showed a neo' clonogenic cell doubling time of 36-42 hours.

Normal PBMCs (10^4 /mL) were spiked with 10% K562-neo' cells, and NK-92 cells were added to yield different effector:target (E:T) ratios of NK-92:K562-neo' cells. (Wong et al. (1996)). Briefly, PBMCs were suspended in

enriched alpha medium (Myelocult™) as described above. This medium has been shown to provide optimal conditions for supporting both IL -2 activation of PBMCs and hematopoietic progenitor cell function (Klingemann et al., *Exp. Hematol.* 21:1263 (1993)). The final concentration of PBMCs in 35-mm tissue culture dishes (Corning, East Brunswick, NJ) was 1×10^6 /mL, and the proportion of input K562-neo' cells was kept at 10% for all experiments. Various numbers of irradiated (1000 cGy) NK-92 cells (see Examples 7 and 8) were added, resulting in various E:T ratios as specified in Table 2. These mixtures were cultured in an atmosphere of 5% CO₂ in air for 4 or 48 hours at 37°C with and without IL-2 (500 units/mL).

After the culture, cells were washed in RPMI/5% FCS, 10^3 cells were suspended in 0.8% Iscove's methylcellulose containing 0.8 mg/mL neomycin, and 1.1-mL volumes were plated in 3-mm petri dishes. After 7 days at 37°C in a humidified atmosphere of 5% CO₂ in air, colonies were counted. The number of neo' colonies provided a measure for the number of surviving clonogenic K562-neo' cells present in the cell suspension originally plated. Percent survival values for co-cultures containing various numbers of NK-92 cells were determined by comparing the number of clonogenic K562-neo' cells present in test co-cultures with the number of those present in control co-cultures (no NK-92 cells added) and harvesting after the same period of incubation. At an input number of 10^3 K562-neo' cells prior to purging, the absolute number of clonogenic K562-neo' cells after 4 hours with no NK-92 cells present was 6400 ± 820 cells and after 48 hours $28,300 \pm 2100$ cells. The mean \pm SEM of four to eight experiments is reported.

When only PBMCs were plated in the neomycin-containing methylcellulose medium, no colonies were ever observed. To quantitate the purging capacity of NK-92 cells, PBMCs were spiked with 10% K562-neo' cells and cultured for 4 or 48 hours in medium in the presence or absence of

IL-2. Results, summarized in Table 2, show that NK-92 cells used at E:T ratios of 10:1 and 5:1 eliminated the K562-neo⁺ cells from PBMCs, and that very low survival was observed at E:T of 1:1. The presence of IL-2 during the purging did not result in any increase in the number of K562 cells purged compared to no IL-2 (Table 2).

Table 2. Purging effect of NK-92 cells

NK-92:K562-neo ⁺ E:T Ratio	% Survival (-IL-2) of K562-neo ⁺		% Survival (+IL-2) of K562-neo ⁺	
	4 hrs	48 hrs.	4 hrs.	48 hrs.
10:1	0	0	0	0
5:1	0	0	0	0
1:1	10.5±2.1	15.4±7.2	6.5±3	15.2±5.9
0.1:1	56±14.1	68.5±19.5	54.4±13	69.6±16.8

Example 6. Effect of NK-92 Cells on Hematopoietic Progenitor

Cells. The effect of NK-92 cells on PBMCs was determined (Cashman et al., *Blood* 75:96 (1990)). Briefly, normal PBMCs were co-cultured with irradiated (1000 cGy) NK-92 cells (see Examples 7 and 8) for 2 days. Cells were then plated in replicate 1.1-mL aliquots of methylcellulose-containing media at densities adjusted to give approximately 10-100 large colonies of erythroid cells (from burst-forming units-erythroid [BFU-E]), granulocytes and macrophages (from colony-forming units-granulocyte/macrophage [CFU-GM]), and combinations of all of these (from CFU granulocyte/erythroid/macrophage/megakaryocyte [CFU-GEMM]). Colonies were counted under an inverted microscope 2 weeks later.

The number and growth kinetics of clonogenic hematopoietic cells were quantified at a 1:1 ratio of NK-92:PBMC after 2 days of co-culture with irradiated (1000 cGy) NK-92 cells. The cells were plated in standard methylcellulose and counted 2 weeks later. Results obtained from three different normal donors are presented as percentage of normal controls in

Table 3. No growth inhibitory effect on hematopoietic progenitors by NK-92 cells was noted.

Table 3. Effect of NK-92 cells on colony formation of normal hematopoietic progenitor cells.

Experiment Number	CFU-GEMM	BFU-E	CFU-C
1	100	46	94
2	200	98	64
3	33	104	103

- Example 7. γ -Irradiation of NK-92 Cells.** NK-92 cells were irradiated in T25 flasks (Corning, Newark, NJ) with the dose indicated using a cesium source (Cis-US, Bedford, MA). A dose range of 200-2000 cGy was tested.
- 5 After irradiation, the cells were washed twice in RPMI, resuspended in medium, and cultured for 72 hours at 37°C in the presence of 500 IU/mL IL-2. Cytotoxicity (^{51}Cr -release assay) was performed with these cells as described above in Example 2. Prior to performing the ^{51}Cr -release assay, the cells were left for 24 hours in medium supplemented with IL-2 to allow for recovery.
 - 10 Proliferation was assessed by means of a ^3H -thymidine incorporation assay. Prior to adding ^3H -thymidine (0.5 $\mu\text{Ci}/\text{cell}$), NK-92 cells were resuspended in thymidine-free RPMI. Uptake of ^3H -thymidine was measured in a liquid scintillation counter 4 hours later. (Klingemann et al., *Leuk. Lymphoma* 12:463 (1994)). The counts per minute (cpm) from three different
 - 15 experiments are presented.

Clinical use of this cell line to purge cancerous cells requires that NK-92 cells not undergo significant growth and proliferation. This was achieved by irradiating the cells. Proliferation, as measured by ^3H incorporation, was effectively reduced at a dose of 1000 cGy (Table 4). The cytotoxicity of NK-92 cells after administration of various radiation doses is presented in Figure 3. At doses up to 1000 cGy an essentially undiminished cytolytic response was maintained.

Table 4. Effect of irradiation on the proliferation of NK-92 cells

Radiation dose (cGy)					
Number	0	500	1000	1500	2000
1	5766	3071	406	125	114
2	4236	2411	1216	1192	562
3	3994	2046	824	689	748

Example 8. Radiation susceptibility of NK-92 cells. NK-92 cells were irradiated by a γ ray source (Gammacell 40, Atomic Energy of Canada. Ltd., Canada). A dose range of 100-3000 cGy was tested. After irradiation, the cells were washed and resuspended in culture medium with rhIL-2. Colony assays, viability and cytotoxic activity of the irradiated NK-92 cells were performed using standard techniques (Yan et al., *Leukemia*, 7:131-139 (1993)). To quantify clonogenic NK-92 cells, NK-92 cells (500 cells per mL culture medium) were cultured in a 0.3% agar-based medium supplemented with 12.5% FCS, 12.5% horse serum, 2 mM L-glutamine, 100 μ g/mL penicillin 50 μ g/mL streptomycin, 10^{-6} M mercaptoethanol, and 500 U/mL rhIL-2 at 37°C for 14 days. An additional aliquot of 500 U/mL rhIL-2 was added at day 7 during the culture. Triplicate cultures were performed for each data point.

The viability of NK-92 cells, determined by trypan blue staining, and the recovery of the ability of the NK-92 cells to generate colonies after exposure to various radiation doses is shown in Figures 4 and 5, respectively. The NK-92 cells maintained substantial survival for 3 or 4 days after exposure to high doses of radiation (1000-3000 cGy). However, *in vitro* clonogenic NK-92 cells were significantly depleted after low doses of radiation and totally eliminated by doses above 300 cGy. Figure 6 shows the cytotoxicity of NK-92 cells to K562, HL60 and 2 patient-derived leukemic samples after exposure to the different doses of radiation. Doses of 300, 500,

and 1000 cGy allow for substantial cytolysis against leukemic cell lines and primary leukemias 1-2 days after radiation.

These experiments suggest that NK-92 cells, irradiated to an extent that renders them nonclonogenic, retain their cytolytic activity against a wide
5 spectrum of target cells. They therefore may be used *ex vivo* in the purging of tumor cells as well as in the treatment of various cancers *in vivo*.

Example 9. Cytolysis of human primary leukemic cells by NK-92.

a. Patient-derived leukemic samples. Samples were obtained, with informed consent, during routine diagnostic blood studies or bone
10 marrow (BM) aspirates from patients with newly diagnosed or relapsed leukemias. 9 acute myeloid leukemia (AML) cases, 11 chronic myeloid leukemia (CML) cases (6 chronic phase, 1 accelerated phase and 4 blast crisis), 14 B-lineage-acute lymphoblastic leukemia (ALL) cases (13 pre-B-ALLs and 1 B-ALL) and 6 T-ALL cases, were studied (see Table 5). Blast-
15 enriched mononuclear cells were isolated by Ficoll Hypaque (Pharmacia, Piscataway, NJ) density gradient separation and washed in RPMI 1640 medium.

b. Effector cells. NK-92 cells were cultured and maintained in α -MEM medium supplemented with 12.5% FCS, 12.5% horse serum and
20 rhlL-2 (500 U/mL Chiron, Emeryville, CA). TALL-104 cells (a MHC-unrestricted human cytotoxic T cell clone, generously provided by Drs. D. Santoli and A. Cesano, The Wistar Institute, Philadelphia) were maintained in Iscove's modified Dulbecco's medium supplemented with 10% FCS and rhlL-2 (100 U/mL) (Cesano et al., *Blood*, 87:393-403 (1996)). Another human NK
25 cell clone, YT, was maintained in RPMI 1640 medium with 10% FCS and rhlL-2 (100 U/mL) (Yodoi et al., *J. Immunol.*, 134:1623-1630 (1985)).

c. Cytotoxicity assays. The cytotoxic activity of non-irradiated NK-92 and responding T cells against leukemic targets was measured in a

standard 4-hour chromium release assay (CRA). Some of the samples were also measured in an 18-hour CRA. A fixed number of ^{51}Cr -labeled target cells (5×10^3 /well) was tested for susceptibility to 4 effector cell concentrations. ^{51}Cr release of target cells alone (spontaneous release, determined by placing target cells in 5% Triton) was always <25% of maximal ^{51}Cr release. CRA data were expressed as specific lysis (%) at a given effector:target (E:T) ratio or were converted to lytic units (LU) defined as the number of effectors resulting in 30% lysis of target cells (Cesano et al., *Cancer Immunol. Immunother.*, 40:139-151 (1995)). The degree of sensitivity of patient-derived leukemic cell targets to each effector was defined as insensitive (-/+; <10/10-19% lysis), sensitive (++/+++/++++: 20-29/30-39/40-49% lysis) and highly sensitive (+++++/+++++: 50-59/>60% lysis) at an E:T ratio of 9:1.

d. Results: Cytolysis of human primary leukemic cells by NK-92 cells. The sensitivity of patient-derived leukemic cells to the cytotoxic effect of NK-92 cells is summarized in column 4 of Table 5. Of the 40 patient-derived leukemic samples shown in Table 5, 26 (65%) were sensitive or highly sensitive to NK-92 mediated *in vitro* cytotoxicity. Six of the samples that were insensitive to the NK-92 cells in the standard 4 hr CRA (sole or first entries), became sensitive after 18 hours incubation (second entries, enclosed in parentheses). Leukemia blasts derived from 6 out of 9 (67%) AML, 6 of 6 (100%) T-ALL and 6 of 14 (43%) B-lineage-ALL were either sensitive or highly sensitive to the NK-92 mediated lysis. 7 of 8 acute leukemia samples which demonstrated high sensitivity to the cytotoxic effect of NK-92 cells were derived from relapsed patients and 1 was from a newly diagnosed patient. Out of 11 CML samples, 8 (73%) were sensitive (5 in chronic phase) or highly sensitive (2 in blast crisis; 1 in accelerated phase) to the NK-92 mediated cytolysis (Table 5).

In comparison, the last two columns in Table 5 present results obtained with cell lines known in the field to have cytolytic activity against tumor cells, namely, TALL-104 cells and YT cells. Only 16 out of the 37 leukemic samples tested (43%) were sensitive (4 AMLs, 5 B-lineage ALLs and 3 CMLs) or highly sensitive (1 AML, 1 B-lineage-ALL and 2 CMLs) to the MHC unrestricted cytotoxic T cell clone TALL-104 mediated cytolysis. Leukemias sensitive to the TALL-104 cells were not consistently sensitive to NK-92 cells, and cells that were lysed by NK-92 cells were not always lysed by TALL-104 cells. In addition, the cytolytic activity of TALL-104 cells was usually detected only after 18 hours of incubation (second entries, enclosed in parentheses). Only four of 16 (25%) of the target samples that were lysed at 18 hours were also lysed in the standard 4 hr CRA. The remaining 12 (75%) responded only after the 18 hour incubation, with the response being generally lower than that observed with the NK-92 cells of the invention. Without wishing to be bound by theory, these observations may be due to the possibility that 1) different target structures are recognized by TALL-104 vs NK-92 cells, or 2) a different pathway may be involved in the NK-92 and in the TALL-104 cell mediated cytolysis.

The majority of leukemic samples treated with YT cells, the other NK-like clone tested, were found to be resistant, with the exception of 2 samples (a CML in blast crisis and a T-ALL) (see Table 5).

In conclusion, the NK-92 cells of the invention are surprisingly and significantly more effective in lysing patient-derived tumor cells, and exert their effect in a shorter time, than do the cells from two cytolytic cell lines known in the field.

Table 5. Cytotoxicity of NK-92, T-ALL104 and YT Clone to Patient-Derived Leukemic Cells^a

Patient	Disease Status	Blast (%) in Sample	Cytotoxic Sensitivity		
			NK-92	TALL-104	YT
AML					
1 M4	Relapse	PB (66%)	++++++	+++++	-
2 (M1)	Relapse	PB (50%)	+++++	..	-
3 (M3)	Relapse	PB (50%)	+++ (++++)	+ (++++)	- (-)
4 (M4)	Refractory	PB (90%)	++ (++)	- (+)	- (-)
5 (M2)	New	BM (90%)	+++ (+++)	+ (+++)	ND
6 (M4)	New	BM (97%)	-	-	-
7 (M4)	New	PB (39%)	- (-)	- (++)	- (-)
8 (M3)	New	PB (55%)	- (++)	- (+++)	+ (-)
9 (M3)	New	BM (32%)	-	-	-
T-ALL					
1	Relapse	BM (98%)	++++++	-	-
2	Relapse	PB (85%)	++++++	- (-)	+++ (+++)
3	Relapse	PB (77%)	++++++	- (+)	- (-)
4	Relapse	PB (60%)	+++++	- (-)	+ (-)
5	New	BM (40%)	+++	-	-
6	New	BM (66%)	+++	-	-
B-Lineage-All					
1 •	Relapse	BM (78%)	+++++	++++	-
2	New	BM (30%)	++++	ND	ND
3	Relapse	BM (75%)	+++ (++++)	+ (++++)	++ (++)
4	New	BM (97%)	++ (+++)	+ (+++)	- (-)
5	Relapse	BM (60%)	+ (+)	- (+)	- (-)
6	Relapse	BM (80%)	-	ND	ND
7	Relapse	PB (80%)	-	- (-)	-
8	New	BM (68%)	-	-	-
9	New	BM (33%)	-	- (+)	-

Patient	Disease Status	Blast (%) in Sample	Cytotoxic Sensitivity		
			NK-92	TALL-104	YT
10	Relapse	BM (87%)	-	- (++)	-
11	Relapse	BM (75%)	- (+++)	- (+++++)	-
12	New	BM (30%)	-	-	ND
13	New	PB 90%)	- (+++)	- (+++)	ND
14	New	BM (81%)	-	-	ND
CML					
1	BC	PB (45%)	+++++	+++++	+++
2	AC	PB (22%)	+++++	++	-
3	BC	PB (93%)	+++++	+	-
4	CP	PB (15%)D	++++	+	-
5	CP	PB (8%)D	++ (++++)	ND	ND
6	CP	BM (12%)D	+ (+++)	+ (+)	ND
7	CP	BM (10%)D	+ (+++)	+ (++++)	ND
8	BC	PB (60%)	+	-	-
9	BC	BM (48%)	+	- (-)	-
10	CP	PB (21%)D	+ (++++)	- (++++)	- (-)
11	CP	PB (11 %)D	-	- (+++++)	- (-)

Notes and Abbreviations. a) Columns show results of chromium release assays at E:T = 9:1 after 4 h without parentheses, and (results after 18 h enclosed in parentheses); New: newly diagnosed; ND: none done; o: FAB classification; D:blast and promyelocyte; BM: bone marrow; PB: peripheral blood; t:B-ALL; BC: blast crisis; AC: accelerated phase; CP: chronic phase.

Example 10. Cytotoxicity of NK-92 towards human leukemic cell lines.

The following human leukemic cell lines were cultured at 37°C in 5% CO₂ in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine and antibiotics: K562 (Chronic myeloid leukemia in blast crisis), HL60 (acute promyelocytic leukemia), KG1 (erythroleukemia), NALM6 (acute pre-B lymphoblastic leukemia), Raji (Burkitt's lymphoma), CEM/S (acute T lymphoblastic leukemic cell line sensitive to methotrexate

(MTX), a commonly used antitumor drug) as well as CEM/T (methotrexate-resistant subline of CEM/S) (Mini, E. et al., *Cancer Res.* 45:325-330 (1985)).

5 NK-92 cells were highly cytotoxic to all the 8 leukemic cell lines tested in a 4 hr standard CRA (Table 6). The MTX-sensitive T-ALL cell line CEM/S as well as its MTX-transport resistant subline CEM/T displayed a similar sensitivity to the NK-92 cells. This suggests that tumors that are not responsive to MTX treatment could be treated by administering NK-92 cells of the instant invention. Table 6 surprisingly shows highly effective cytolytic activity for NK-92 against all the target cells tested. The results obtained at
10 the low E:T ratio of 1:1 are especially noteworthy.

In contrast, the cytolytic cell lines TALL-104 and YT have little or virtually no cytolytic activity against many of these target cells under these conditions; when active, their activity is generally lower than that for NK-92 at E:T of 1:1. TALL-104 was cytotoxic to K562, NALM6 and HL60 cells,
15 however, Raji cells exhibited only 22.2% lysis at 9:1 E:T ratio and KG1 cells, CEM/S as well as CEM/T were resistant. The YT clone did not exhibit significant cytotoxic activity. Activity was found only against K562 cells and Raji cells, which showed a 32% and 25% lysis at 9:1 E:T ratio, respectively.

As shown in Table 6, NK-92 cells of the present invention have a
20 significantly wider range of action and higher activities than the known cytolytic cell lines TALL-104 and YT. These activities are higher than any previously reported values in the field of tumor cytototherapy.

Table 6. Specific Lysis of Human Leukemia Cell Lines by Natural Killer Cell Clones NK-92, TALL-104, and YT.

Target				Specific Lysis (%)					
	NK92			TALL-104			YT		
	Effector: Target Ratio								
	9:1	3:1	1:1	9:1	3:1	1:1	9:1	3:1	1:1
K562	94.1	91.2	82.1	88.5	85.2	72.5	34.2	28.2	18.4
HL60	87.9	75.3	79.6	43.0	16.0	6.9	2.1	1.1	1.5
KG1	64.6	53.8	43.7	2.7	0.5	0	0.1	0	0
NALM6	72.6	56.8	52.4	67.8	55.6	33.3	1.0	0.5	0
Raji	86.0	75.4	70.0	22.2	10.2	0.3	25.1	18.0	14.2
TALL-104	57.3	53.2	44.1	-	-	-	32	1.4	0.9
CEM/S	56.6	48.8	34.7	2.7	1.6	0.9	0.9	0.4	0.3
CEM/ T	57.5	42.1	39.1	1.5	0.6	0.3	1.2	0.1	0.2

Example 11. Effect of NK-92 cells on normal human bone marrow

hematopoietic cells. Heparinized bone marrow collected from normal

- 5 donors was separated by Ficoll Hypaque density gradient isolation to produce the mononuclear cells. Enrichment of hematopoietic cells and depletion of T cells was achieved by soybean lectin agglutination (SLA) of mature marrow elements and removal of residual T cells by resetting with sheep red blood cells (Reisner et al., *Lancet*, 2:327-31 (1981)).

- 10 Hematopoietic cell enriched fractions of normal bone marrows from 14 normal donors were tested by standard CRA to determine their susceptibility to lysis by NK-92 cells. All of the normal bone marrow samples were insensitive to NK-92 mediated cytotoxicity (Figure 7).

Example 12. In vivo leukemogenesis of NK-92 cells in SCID mice.

- 15 a. **Experimental animals.** Severe combined immunodeficient (SCID) mice (CB17 scid/scid and pfp/Rag-2) (6 to 8 weeks old; Taconic Farms, Germantown, NY) were maintained in microisolator cages under

sterile conditions with a specific pathogen-free environment. To determine the potential of NK-92 cells to induce leukemia *in vivo*, 2×10^7 viable NK-92 cells in 0.3 mL phosphate buffered saline (PBS) were administered by either intraperitoneal (I.P.) or intravenous (I.V.) route every other day for 5 injections in each animal. For subcutaneous (S.C.) inoculations, 2×10^7 NK-92 cells were injected in the right flank of SCID mouse, as described previously (Yan et al., *Blood*, 88:3137-3146 (1996)). Thereafter, all the experimental animals were administered rIL-2, 5×10^4 U every other day for 2 weeks by S.C. injection. Survival of the animals was followed for at least 6 months after inoculation.

b. Tissue analysis. From each group, 2 SCID mice were sacrificed at the end of observation and tissues from peripheral blood, bone marrow, spleen, liver, kidney, lung, and brain were collected for histopathological and/or fluorescence activated cell sorting (FACS) analysis. Tissue sections from sacrificed SCID mice were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin, sectioned and stained according to standard histological techniques.

Viable cells recovered from various tissues were stained by fluorescein isothiocyanate-conjugated (FITC) or phycoerythrin-conjugated (PE) Mab, as described (Yan et al., (1996)). A FACS scan flow cytometer (Becton Dickinson) was used for analysis. Monoclonal antibodies (Mabs) directed against the respective human cell surface antigens were used for determination of their presence: CD2, CD3, CD5, CD7, HLA-DR, CD45, CD56 (Becton Dickinson). A fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse Mab mCD45 (Boehringer-Mannheim, Indianapolis, IN) was used for characterization of murine leukocyte common antigen.

c. Leukemogenesis. CB-17 scid/scid mice as well as pfp-Rag-2 mice were inoculated with NK-92 cells by I.V. ($n = 3$, for each group), S.C. (n

= 2, each group) and I.P. (CB-17: n = 8; pfp-Rag-2: n = 3) injection. Survival of the animals was followed at least 6 months after inoculation. At the end of the six month period, all animals appeared healthy; there was no hepatosplenomegaly, lymphadenopathy or leukemic nodular growth, which would have indicated leukemia development. Leukemic cellular infiltration was not detected in the different tissues of the sacrificed animals by histopathology. No cells of human origin were detectable in the tissues by FACS analysis.

Example 13. Comparison of antileukemic effect of NK-92 cells

with LAK, NK and T cells against human leukemic cell lines. To isolate the NK cell populations, a Cephate^R cell separation system based on avidin-biotin immunoaffinity (CellPro, Bothel, WA) was used to purify a CD56+ cell fraction from cultured LAK cells. Briefly, the harvested cells were washed and resuspended in PBS with 1% bovine serum albumin (BSA). To each 1-
2x10⁸ cells/mL, 40µL primary monoclonal antibody (mouse anti-human CD56) was added and the cells were incubated at 4°C for 25 minutes. After incubation, the cells were washed and resuspended to a concentration of 1x10⁸ cells per mL in PBS with 1% BSA. Then, to each one mL cell suspension, 20µL biotin labeled rat antimouse IgG1 antibody was added and the cells were incubated again at 4°C for 25 minutes. After incubation, the cells were washed and resuspended at a concentration of 1x10⁸ cells per mL in PBS with 5% BSA and slowly passed through the avidin column. The CD56+ cells were captured; other cells, including the T cell fraction, were eliminated from the column. After washing the column, the adherent cells were then disassociated from the column by agitation and elimination. After separation, the NK cell-enriched populations contained >85% CD56+CD3- NK cells. The majority of the other cells in the fraction (>95%) were CD3+CD56- T cells.

To generate leukemia-reactive alloctotoxic T lymphocytes (CTLs), peripheral blood mononuclear cells (PBMC) isolated from normal donors were cultured with irradiated leukemic stimulating target cells and irradiated autologous PBMC as feeder cells. Cultures were started in 60 well plates at 5 1000 responder cells per well in RPMI 1640 medium containing 15% human serum and rIL-2 100 U/mL at 37°C, 5% CO₂. The ratios of stimulator cells and feeder cells to responder cells were 5:1 and 10:1, respectively. After 10-12 days culture, CTLs were harvested from growth-positive wells and specific lysis toward leukemic target cells and K562 cells was quantitated by ⁵¹Cr-10 release assay. The CTLs were continuously cultured and fed with stimulator and feeder cells in flasks. After 2-3 weeks culture, the monoclonal antibody OKT3 (Ortho Biotech, Raritan, N.J.) was added to the culture for rapid expansion of the CTL lines.

The antileukemic effects of NK-92 cells, human LAK cells, NK cells 15 (CD56+ CD3-; CD56+ in Figure 8), and T cells (CD3+CD56-; CD3+ in Figure 8) were assessed by measuring *in vitro* cytolytic activity in standard CRA (Figure 8, Panels A and B), and by measuring inhibition of leukemic cell xenograft growth *in vivo* (Figure 8, Panels C and D) when the effector cells and targets were co-inoculated subcutaneously into SCID mice. In order to 20 evaluate the inhibition of growth *in vivo*, the area of the subcutaneous growths of leukemic nodules as a measure of their size was determined once a week after inoculation, and survival of the animals was also followed. NK-92 cells displayed the highest *in vitro* cytotoxicity against K562 (Figure 8, Panel A) and HL60 (Figure 8, Panel B) of the cells tested, with a mean specific lysis of 25 89% and 78%, respectively. This was superior to the killing mediated by human LAK (52% and 11%, respectively), NK (72% and 28%, respectively) and T cells (12% and 1.2%, respectively).

Correspondingly, the NK-92 cells demonstrated more effective *in vivo* inhibition of the growth of K562 (Figure 8, Panel C) and HL60 (Figure 8, Panel D) leukemic cells xenografts than did the human LAK and NK cells. The results shown in Figure 8 indicate that the NK-92 cells of the present invention have cytolytic activity *in vitro* and tumor-inhibiting activity *in vivo* that is superior to those activities manifested by the known preparations of cytolytic cells normally present in humans. These activities are therefore unexpected by a worker in the field of tumor cytototherapy.

Example 14. Comparison of antileukemic effect of NK-92 cells with allogeneic leukemic-reactive CTL cells. To examine the *in vivo* effects of NK-92 cells and other effector cells on the growth of human leukemia xenografts, 5×10^6 leukemic target cells alone or mixed with 2×10^7 NK-92 or other effector cells (E:T ratio=4:1) were injected S.C. into SCID mice. The TALL-104 effector cells were irradiated with 3000 cGy before inoculation to prevent leukemogenesis in SCID mice. RhlL-2 was administered to the mice, as in Example 13. The Log-rank test and Wilcoxon test were used for the comparison of the survival of leukemia bearing SCID mice.

The antileukemia effect of NK-92 cells was evaluated using allogeneic leukemia-reactive CTL cells (derived from a patient with T-ALL (TA27)). Both NK-92 and CTL cells activated by exposure to TA27 displayed a significantly higher specific cytolysis (70% and 58% at 9:1 E:T ratio, respectively) than the other effectors (LAK cells: 22%; NK cells (designated CD56+ in Figure 9): 38%; TALL-104: 8%; and T cells (CD3+ in Figure 9): 1.5% specific lysis) against the TA27 leukemic cells (Figure 9, Panel A). Correspondingly, the subcutaneous growth of TA27 leukemic cells was inhibited by co-injection of either NK-92 cells or anti-TA27-CTL cells (Figure 9, Panel B). The survival of those animals which were co-inoculated with TA27 leukemic cells plus NK-92

or with anti-TA27-CTL cells was significantly prolonged beyond that of the animals bearing TA27 leukemia alone (NK-92 cells: $p=0.001$; TA27-CTL cells: $p=0.002$; see Figure 10). In contrast the TALL-104 cells did not show significant *in vitro* killing against TA27 leukemic cells by CRA (Figure 9, Panel A). However, moderate inhibition of the leukemic tumor growth *in vivo* (Figure 9, Panel B), coupled with a statistically insignificant ($p>0.05$) increase in survival, was observed in the animals co-inoculated with TA27 leukemic cells and irradiated TALL-104 cells (Figure 10).

Example 15. Antileukemia effect of NK-92 cells in human leukemia xenograft SCID mice model. For study of the *in vivo* tumoricidal capacity of NK-92 cells, leukemic cells derived from a T-ALL patient (TA27), an AML patient (MA26), and a pre-B-ALL patient (BA31) were adoptively grown and expanded in SCID mice by S.C. inoculation. Leukemic cells recovered from the leukemic nodules in the mice (first passage) were used in these experiments. The SCID mice in each group were inoculated I.P. with 5×10^6 leukemic cells from the first passage in 0.2 mL PBS, and 24 hours later 2×10^7 NK-92 cells in 0.4 mL PBS were administered by I.P. injection. The animals received either 1 dose or a series of 5 doses of NK-92 cells which were administered on days 1, 3, 5, 7, and 9, with and without rIL-2, as indicated in the Figures.

All the human leukemias grew aggressively in SCID mice. Leukemic cells derived from a patient (TA27) with T-ALL and a patient (MA26) with AML M4 leukemia were highly sensitive *in vitro* to the NK-92 cells (73% and 66% specific killing at 9:1 E:T ratio determined by CRA, respectively), whereas cells from a patient with pre-B-ALL (BA31) were insensitive to the NK-92 cells (4% specific killing at 9:1 E:T ratio assessed by CRA). Figure 11 shows that the survival of mice bearing TA27 leukemia was significantly prolonged by the administration of NK-92 cells. The median survival time (MST) of the

animals with no treatment or rhIL-2 alone was 72 days (n=5) and 63 days (n=5) ($p>0.05$), respectively. All these animals died of leukemia. Treatment with NK-92 cells (alone or with rhIL-2) increased the MST to 102 days (n=6) and 114 days (n=6), respectively, for the 1 dose injection schedule (2×10^7 NK-92 cells, day 1). The MST increased to 160 days (n=6) and 129 days (n=6), respectively, with 5 doses NK-92 with or without rhIL-2 injection (Figure 11). Three animals that received 5 doses of NK-92 cell injections with or without rhIL-2 administration survived without any signs of leukemia development 6 months after inoculation. There was no significant difference in survival between the mice receiving treatments with or without rhIL-2 administration, whether in the group receiving 1 dose of NK-92 cells ($p=0.75$), or the in the group receiving 5 doses ($p=0.45$). Compared to the group receiving 1 dose of NK-92 cells, with or without rhIL-2 treatment, survival was significantly extended in animals that received 5 doses of NK-92 cells without rhIL-2 treatment ($p=0.009$ and $p=0.009$, respectively).

In SCID mice inoculated with human pre-B-ALL (BA31) leukemia, with or without rhIL-2 treatment, the MST were 63 days (n=5) and 64 days (n=5), respectively (see Figure 12). For the animals that received 5 doses of 2×10^7 NK-92 cells, with or without rhIL-2 administration, the MST was increased to 79 days (n=5) and 76 days (n=5), respectively. These survival times were not significantly different from those for the animals that were not treated by NK-92 cells ($p>0.05$).

In animals bearing human AML (MA26), MST was 97 days (n=6) (see Figure 13). The MST was extended to 173 days among the animals that received 5 doses of 2×10^7 NK-92 cells ($p<0.01$) (n=6). Three of the 6 animals that received NK-92 cells remained alive 6 months after leukemia inoculation. Two of these appeared healthy without any signs of leukemia development. One mouse had an enlarged abdomen indicating residual leukemia. The 6

animals that received NK-92 cells plus rhIL-2 treatment were all alive 6 months after leukemia inoculation without any signs suggestive of leukemia development.

The results presented in Figures 11-13 show that *in vivo* treatment of
5 leukemic tumors can result in enhanced longevity of the subject mice. The
extent of the prolongation of life, and of the improvement in the health of the
animals, is dependent on the particular leukemic tumor involved, and ranges
from modest or insignificant (Figure 12) to very dramatic (Figure 13). Based
on these results, it is concluded that treatment of tumors *in vivo* by
10 administering NK-92 cells, depending on the tumor in question, can be
surprisingly effective.

**Example 16. Preparation of Modified NK-92 Cell Lines Secreting
IL-2.** In order to generate NK-92 cells that constitutively secrete IL-2, two
plasmids encoding human IL-2 were employed.

15 a. **Methods.** DNA Clones: The MFG-hIL-2 vector (Figure 14) was
generously provided by Dr. Craig Jordan (formerly of Somatix Corp.,
Alameda, CA). The pCEP4-LTR-hIL-2 vector (Figure 15) was created by
excising the *Hin* DIII-Bam HI fragment from the MFG-hIL-2 vector, containing
the 5' LTR and hIL-2 gene, and inserting it into the complementary sites of the
20 pCEP4 episomal vector backbone (InVitrogen, Carlsbad, CA).

Particle-Mediated Gene Transfer: NK cells were transduced by
particle-mediated gene transfer using the Biolistic PDS-1000/He Particle
Delivery System (BioRad Laboratories, Hercules, CA). Cells were transduced
according to the manufacturer's instructions. Briefly, 1.0 or 1.6 μ m gold
25 particles were coated with 5 μ g of DNA using calcium chloride spermidine,
and ethanol. NK-92 cells were prepared for bombardment by adherence to
poly-L-lysine (Sigma, St. Louis, MO) coated 35mm tissue culture plates.
Cells were bombarded in an evacuated chamber (vacuum of 20 inches

mercury) and DNA-coated particles were accelerated by a 1,100 psi helium pulse. Cells were returned to IL-2 supplemented Myelocult media immediately following bombardment and allowed to recover for 24 hours prior to transfer to IL-2-free media. Media was changed periodically. Cells were
5 selected for IL-2-independent growth. Preliminary experiments showed heat transfer efficiencies of 5-15% were obtained under the conditions used.

PCR and Southern Blot Analysis: The transfection of the NK-92 cells was confirmed by polymerase chain reaction (PCR) analysis of DNA isolated from both the parental and transfected NK-92 cell lines for the
10 presence of genomic and cDNA forms of the human IL-2 gene. DNA was isolated using DNAzol (Gibco Life Technologies Inc., Burlington, ON). Briefly, cells were lysed in DNAzol and DNA was precipitated with ethanol at room temperature. DNA pellets were collected, washed in 95% ethanol and briefly air dried. DNA was resuspended in 8 mM NaOH at 62°C and
15 solution was neutralized with HEPES buffer. DNA was quantitated by absorbance at 260 nm. Primers flanking exon 1 of the human IL-2 gene (forward: 5'-CAA CTC CTG TCT TGC ATT GC-3' (SEQ ID NO:1) and reverse: 5'-GCA TCC TGG TGA GTT TGG G-3' (SEQ ID NO:2), Gibco Lift Technologies Inc., Burlington, ON) were used to amplify the DNA (30 cycles,
20 1 min 95°C, 2 min 50°C and 2 min 72°C). PCR products were resolved on a 2% agarose gel. For Southern blot analysis, DNA was transferred to Hybond + nylon membrane (Amersham Life Sciences, Arlington Heights, IL) by capillary transfer in 10 X SSC (1.5M NaCl, 1.5M NaCitrate) and fixed by UV cross-linking (StrataLinker Stratagene, La Jolla, CA). The blot was hybridized
25 with a ³²P radiolabeled human IL-2 probe for 8-12 hours, washed and visualized by autoradiography at -70°C with Kodak X-Omat XAR film.

Northern Blot Analysis: Cytokine and chemokine gene expression was analyzed by Northern blot analysis. RNA was extracted from

parental and transfected NK-92 cell lines using Trizol reagent (Gibco Life Technologies Inc., Burlington, ON) according to the manufacturer's instructions. Briefly, cells were lysed in Trizol and the lysate extracted with chloroform. The aqueous phase was then precipitated with isopropanol. The
5 RNA pellet was collected, briefly air-dried and then resuspended in DEPC-treated water (diethyl-pyrocabonate; Sigma Chemical Co., St. Louis, MO). RNA was quantitated by determining OD₂₆₀. Fifteen micrograms of RNA was resolved on a 1% formaldehyde agarose gel in 1% MOPS (3-[N-Morpholino]propanesulfonic acid, Sigma, St. Louis, MO) and blotted as
10 described previously for Southern blot analysis. The blot was hybridized with ³²P radiolabeled probes for human IL-2 and TNF- α .

DNA probes for Northern and Southern blot analysis were radiolabeled by random primer extension. DNA probes for human IL-2 and TNF- α were purified by digestion with appropriate restriction endonucleases
15 and agarose electrophoresis. The DNA was excised from the gel and purified by centrifugation through a Spin-X tube filter (Corning Costar, Cambridge, MA), phenol:chloroform extraction and ethanol precipitation. DNA probe was labeled with a ³²P-dCTP (Sp. Ac. 3000 Ci/mmol; ICN, Montreal, PQ).

Cytokine Determination: IL-2 production by NK-92 cell lines was
20 determined by ELISA. Aliquots of 1×10^6 of the parental or transfected NK-92 cells were cultured in 8 ml of IL-2 free Myelocult media for 1, 2, and 3 days. Supernatants were collected from at -20°C until all samples were collected. Samples were thawed and assayed for IL-2 levels by ELISA according to the manufacturers' instructions (Quantikine; R&D Systems, Minneapolis, MN).
25 The ELISA is a horseradish peroxidase/tetramethylbenzidine based colorimetric assay and the ELISA microtiter plates were read at 450 nm (with a 540 nm correction) in a microplate reader (Model EK309, Bio-Tek Instruments Inc., Winooski, VT).

Irradiation of NK-92 Cells: To determine the sensitivity of both parental and transfected NK-92 cells to irradiation, cells were irradiated using a Cis BioInternational 437c cesium source (Cis-US, Bedford, MA). Cells were collected, washed and resuspended in medium and irradiated in 15 or 50 ml conical centrifuge tubes (Becton Dickinson, Franklin Lakes, NJ). Following irradiation, cells were washed and resuspended in Myelocult with (for parental NK-92) or without (for transfected cells) IL-2. Cells were cultured for 24, 48 and 72 hours and assayed for viability by trypan blue exclusion, for proliferation by ^3H thymidine incorporation and for cytotoxicity by ^{51}Cr -release assay (as described above).

- b. Plasmid MFG-hIL-2. For NK cells transfected with the MFG-hIL-2 vector, 85-95% of cells died after 4-7 days following transfer to unsupplemented media. A small number of cells, however, remained viable. These were assumed to be cells that had been successfully transfected.
- However, even with these cells, no viable cells were detectable after two to three weeks. This was expected as the MFG-hIL-2 vector construct did not contain the genetic elements required for replication and maintenance in eukaryotic cells such as a mammalian origin of replication. Therefore, as the transfected cells were maintained in culture and began to replicate, the vector construct would have been lost from cells and the cells would have reverted

to their IL-2-dependent phenotype. These cultures were nevertheless propagated for several weeks. Surprisingly, a small number of viable cells appeared in the cultures after approximately 4-5 weeks following initial transfer of the cells to IL-2-free media. These cells were capable of IL-2-
5 independent growth upon subculturing to fresh media and appeared to be stably transfected, maintaining their IL-2 independent phenotype during prolonged culturing. Since the vector was unable to replicate, the appearance of stably transfected cells suggests that the vector had integrated into the genome of a transfected cell. Since this would be a very rare event,
10 these transfected cells probably arose from one or a very small number of cells. IL-2-independent NK-92 cells arising from transfection with the MFG-hIL-2 were denoted as NK-92MI.

c. Plasmid pCEP4-LTR.hIL-2. Initial observations for cells transfected with the episomal vector pCEP4-LTR.hIL-2 were identical to those seen with
15 NK-92MI. The majority of the transfected cells died within 4-7 days following transfer to IL-2-free Myelocult media. However, unlike the NK-92MI cells, the remainder of the cells did not lose their IL-2-independent phenotype or vitality and die after the initial 2-3 week period. Instead, the cells that were initially
20 IL-2-independent were immediately capable of long-term IL-2-independent growth and survival. This was expected since the pCEP-LTR.hIL-2 vector contains elements that enable it to be maintained in eukaryotic cells as an autonomously replicating genetic element. Therefore, any cell that was initially transfected should maintain its IL-2-independent phenotype for an indefinite length of time. Although cells harboring episomal vectors are not
25 stably transferred by strict definition, these cells are under constant selection pressure in IL-2-free media in favor of cells maintaining the vector. Therefore, these cells are capable of long-term culturing: IL-2-independent NK-92 cells arising from transfection with the pCEP4-LTR.hIL-2 are denoted as NK-92CI.

To confirm that NK-92MI and NK-92CI had in fact been transfected with hIL-2 gene, PCR analysis was performed on the parental and transfected cell lines. Primers flanking exon 1 of the hIL-2 gene, which has 88 base pairs (bp), were used to amplify DNA isolated from NK-92, NK-92MI and NK-92CI
5 to assay for the presence of the genomic and cDNA forms. Agarose gel electrophoresis of the PCR products from the parental line revealed a single 263 bp fragment corresponding to the size expected for the DNA fragment amplified from the genomic IL-2 gene. However, analysis of both the NK-92MI and NK-92CI products revealed two bands, the 263 bp fragment
10 corresponding to the genomic hIL-2 gene as well as a 175 bp fragment resulting from the amplification of the hIL-2 cDNA. To confirm the identity of these DNA fragments, Southern blot analysis with a radiolabeled probe specific for hIL-2 was performed. Both the 263 bp genomic fragment and the 175 bp cDNA fragment hybridized with the probe. These data indicate that
15 both NK-92MI and NK-92CI had been successfully transfected and contained the cDNA for hIL-2.

d. Analysis of Gene Expression. Expression of specific cytokines in the parental and transfected cell lines, they were analyzed by Northern blot analysis. RNA isolated from NK-92, NK-92MI, and NK-92CI cells was
20 separated by electrophoresis, transferred to a nylon membrane and hybridized with probes for the cytokines hIL-2 and hTNF- α . Northern blot analysis of IL-2 revealed that IL-2 RNA was not detectable in the parental cell line. However, hIL-2 RNA was detected in both NK-92MI and NK-92CI. Two mRNA transcripts were detected in NK-92MI cells: a major RNA species of
25 approximately 1.9 kDa and a less intense transcript at 2.4 kDa. In NK-92CI, a hIL-2 mRNA transcript of approximately 1.4 kDa was detected. A very faint band was also seen at 2.5 kDa. These data confirm that the transfected cells expressed IL-2 while the parental NK-92 cells did not. The significance of the

multiple hIL-2 mRNA transcripts in the two transfectants is not clear, although it is possibly a consequence of the different vector constructs. Furthermore, in the case of NK-92MI, the integration of the hIL-2 gene into the genomic DNA may also have affected the RNA size.

- 5 TNF- α expression in the NK cells was also examined using this technique. It was seen that all three lines expressed the gene for this cytokine. A TNF- α probe hybridized to a 1.6 kDa band in RNA isolated from NK-92, NK-92MI and NK-92CI. These results indicate that although transfection of NK-92 cells with the IL-2 gene resulted in expression of the IL-
 10 2 in the transfectants, this did not influence the expression of another cytokine.

- e. Secretion of hIL-2. After confirming expression of the IL-2 gene by Northern blot analysis, cells were assayed for production and secretion of hIL-2 by ELISA. Aliquots of 10^6 NK-92, NK-92MI and NK-92CI cells were plated
 15 in 8 mL aliquots and cultured in Myelocult in the absence of IL-2. Supernatants were collected after 24, 48 and 72 hours for IL-2 analysis by ELISA. Background levels of IL-2 were detected in the supernatant of NK-92 cells at all three time points (2-3 pg/mL). Elevated IL-2 levels were detected
 20 in both NK-92MI and NK-92CI supernatants (Table 7). NK-92MI produced much higher levels of IL-2 in comparison to NK-92CI, with levels ranging from 60x higher after 24 hours (9.3 pg/mL vs 549.3 pg/mL) to about 80x higher after 48 hours (15.7 pg/mL vs 1,260.3 pg/mL) and 72 hours (27.2 pg/L vs 2,248.3 pg/mL).

Table 7: Synthesis of Human IL-2 by NK-92, NK-92MI, and NK-92CI

		IL-2 (pg/ml) in Experiment			
		#1	#2	#3	Ave \pm S.D.
NK-92	Day 1	0	7	1	2.7 \pm 3.8
	Day 2	0	4	1	1.7 \pm 2.1
	Day 3	0	3	3	2.0 \pm 1.7

NK-92MI	Day 1	517	568	545	549.3 ± 34.7
	Day 2	977	1462	1342	1260.3 ± 252.6
	Day 3	1872	2610	2263	1148.3 ± 3692
NK-92CI	Day 1	7	13	8	9.3±3.2
	Day 2	14	16	17	15.7 ± 1.5
	Day 3	52	18	13	27.7±21.2

- f. Comparison of cell surface antigens in NK-92, NK-92MI and NK-92CI. To compare the IL-2-independent transfectants with the parental cells, NK-92MI and NK-92CI were analyzed for CD2, CD3, CD4, CD8, CD10, CD16, CD28, CD56, ICAM-1, ICAM-2, ICAM-3 and LFA-1 expression by fluorescent activated cell sorting (FACS) analysis. The transfected cells revealed a pattern of expression identical to that seen on the untransfected parental cell line with the exception of the IL-2 receptor. FACS analysis of CD25 (the IL-2 receptor α -chain) on NK-92 cells indicated that the receptor was expressed on the surface of NK-92 cells and that its expression is down-regulated in response to IL-2. This confirmed similar findings obtained in earlier work (Gong et al., 1994). Therefore, NK-92 cells in unsupplemented media had relatively high levels of CD25 on their surface while cells in media supplemented with as low as 100 U/mL had low levels of CD25 cell surface expression.
- CD25 expression in the high IL-2-producing transfectant NK-92MI was decreased both in unsupplemented media and in media supplemented with 100 U/mL or 1000 U/mL of IL-2. These results are consistent with those seen with the parental cells. Since the levels of endogenously produced IL-2 in NK-92MI were high, down-regulation of IL-2 receptor levels is expected even in the absence of exogenously administered IL-2.

Culture of NK-92CI in media supplemented with 100 U/mL and 1000 U/mL IL-2 resulted in CD25 upregulation and increased cell surface expression. However, the results for NK-92CI in unsupplemented media are not as clear. Two distinct populations appear, a population expressing very low CD25 levels, similar to NK-92MI, and a population expressing high levels, similar to the NK-92 parental cells. This suggests that NK-92CI consists of a polyclonal population consisting of high and low IL-2 expressing cells rather than a uniform population of cells expressing an intermediate to low level of IL-2. Therefore, when cultured in IL-2-free media, the cells expressing high levels of IL-2 would have low surface levels of CD25 while low IL-2 expressing cells would have high CD25 levels on their surface.

Example 17. Cytotoxicity of NK-92 Transfected to Produce IL-2.

To evaluate the cytotoxicity of these transfected cells, a standard 4 hour ⁵¹Cr-release assay was performed to compare the toxicity of the parental cells to NK-92MI and NK-92CI to the standard test target cells K562 and Raji. The cytotoxicity of NK-92MI and NK-92CI was comparable to that seen with the parent cells (Figure 16). The transfected cell lines show cytotoxic activities against K562 and Raji that are very similar to that of the parental cells. Cytotoxicity of NK-92 against K562 ranged from 82 to 67% while NK-92MI and NK-92CI had cytotoxicity ranges of 77 to 62% and 82 to 62%, respectively. For Raji cells, NK-92 had cytotoxicity of 81 to 47%, NK-92MI had cytotoxicity of 75 to 65% and NK-92CI had cytotoxicity of 82 to 52%.

Example 18. Effect of Transfected NK-92 Cells on Hematopoietic Progenitor Cells. One potential clinical application of the NK-92, NK-92MI and NK-92CI cells is as an *ex vivo* purging agent for autologous grafts. In order for the NK cells to be suitable for such a purpose, they must be able to purge the malignant cells without killing the hematopoietic progenitor cells in the graft or influencing their hematopoietic potential. In order to assay this, a

colony-forming cell assay (CFC) was performed where the clonogenic output of PBMCs was examined following a 48 hour incubation with NK-92MI and NK-92CI at various E:T ratios. NK-92 was previously shown to have minimal effect on hematopoietic stem cells (Example 6). In this example, NK-92MI and NK-92CI also show little or no effect on clonogenic output. The number of total colonies following incubation with either NK-92MI or NK-92CI was very similar to control, although a slight decrease was seen with the highest effector:PBMC ratio of 1:1 (Figure 17). Total clonogenic output from both NK-92MI and NK-92CI was approximately 80% of control under this condition. However, no consistent trend was seen in terms of clonogenic output with respect to the ratio of NK:PBMCs. In terms of specific colony types, there were no detectable differences in the number of output BFU-E colonies, which are the most numerous. Some effect was seen with both the CFU-GM and CFU-GEMM colonies. However, the absolute numbers of these colonies are very low, making any conclusions difficult since small variations in the number of colonies has a large effect on the calculation of clonogenic output. An influence on CFU-GM and CFU-GEMM is seen at higher ratios, but no consistent correlation between ratio and output was noted.

Example 19. Irradiation of the Transfected NK-92 Cells. To

establish an effective irradiation dose to inhibit proliferation and maintain cytotoxicity, NK-92MI and NK-92CI cells were irradiated at 500, 1,000, 1,500 and 2,000 cGy and assayed for proliferation by the ³H thymidine incorporation assay (see Examples 7 and 8). Both NK-92MI and NK-92CI were more sensitive to irradiation than the parental NK-92 cell. Proliferation of NK-92MI and NK-92CI was found to be more strongly suppressed than NK-92 at all radiation doses tested (Figure 18, Panel A). For NK-92MI and NK-92CI, proliferation was completely suppressed by a radiation dose between 500 and 1,000 cGy. The level of thymidine incorporation reached a plateau

at approximately 20% of unirradiated control cells for NK-92CI and 10% for NK-92MI. For determination of viability, NK-92, NK-92MI and NK-92CI cells were irradiated at 250, 500, 1,000 and 2,000 cGy and trypan blue exclusion was determined 24, 48 and 72 hours following irradiation. It was found that greater percentages of both NK-92MI and NK-92CI were found to be killed by irradiation as compared to the parental cells at equivalent doses (Figure 18, Panel B). Viability of NK-92 was higher than that of both transfectants at all dose rates tested.

The cytotoxicity of these cells following irradiation is shown in Figure 19. Cells irradiated at 0, 1,000 and 2,000 cGy were tested after three days for cytotoxicity against K562 and Raji cells at effector:target ratios of 20:1, 10:1, 5:1 and 1:1. Cytotoxicity of NK-92 cells three days following irradiation at 1,000 cGy was determined to be approximately 10-30% K562 (Figure 19, Panel A) and 30-50% and for Raji (Figure 19, Panel B). Irradiation at 2,000 cGy resulted in cytotoxicity of 1-5% against K562 and 3-13% against Raji. In contrast, NK-92MI had only 0-5% and 0-1% cytotoxic activity against K562 and 0-1% and 0% against Raji three days after irradiation doses of 1,000 and 2,000 cGy, respectively. NK-92CI had only 1-4% cytotoxicity to K562 and 2-7% to Raji three days after irradiation at 1000 cGy and 0% to K562 and 0-2% after irradiation with 2000 cGy.

In the data reported here, IL-2 transfectants are seen to be more sensitive to irradiation than the parental strain. Proliferation and cytotoxicity of both NK-92MI and NK-92CI cells were suppressed at a lower radiation level than for the parental strains, and radiation-induced lethality was much greater in the IL-2-independent modified cells in NK-92 at equivalent radiation doses. The high IL-2-producing NK-92MI is more sensitive to radiation than the low IL-2 producing NK-92CI variant. As a result of the increased radiation sensitivity, a reduced level of irradiation would be

sufficient to adequately control proliferation while minimizing lethality to the cells and inhibition of cytotoxicity. In routine experiments, the worker of ordinary skill would be able to repeat experiments such as those described in this example. By using lower radiation doses, in the range between 0 and
5 1000 cGy optimal doses can be determined that inhibit proliferation while maintaining viability and cytolytic activity in NK-92MI and NK-92CI.

Example 20. Transfection of NK-92 with a gene for thymidine kinase. NK-92 cells are to be transfected with a vector bearing a gene for thymidine kinase (TK). The resulting TK-modified NK-92 cells are thereby
10 rendered susceptible to the toxic effects of the guanosine analogs, gancyclovir, and acyclovir.

A vector suitable for transfecting a mammalian cell is to be constructed, such as a retroviral vector harboring a herpes simplex virus (HSV) TK gene, under the control of the HSV TK promoter, and containing its
15 own polyA addition site. Transfection is to be carried out by a method known to those skilled in cell biology and mammalian molecular biology, such as by electroporation (Bio-Rad Gene Pulser™), or by lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413 (1987)). The transfected NK-92 cells so produced are susceptible to inactivation by administering gancyclovir or
20 acyclovir.

Example 21. Mutation of NK-92 HLA cell surface protein. NK-92 cells are to be obtained from the cell line described by Gong et al. (1994). The chromosome bearing the β_2 -microglobulin gene is to be isolated, and the DNA contained within this chromosome is to be purified away from histones
25 and other DNA-bound proteins. The gene fragment bearing β_2 -microglobulin is to be excised with restriction nucleases, and site specific mutagenesis is to be conducted via an oligonucleotide cassette harboring the mutated nucleotide sequence. These procedures employ techniques commonly

known in recombinant DNA technology, as set forth, for example, in "Current Protocols in Molecular Biology", Ausubel et al., John Wiley and Sons, New York 1987 (updated quarterly), and "Molecular Cloning: A Laboratory Manual", 2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989, incorporated herein by reference. The mutated β_2 -microglobulin is to be reincorporated into the cellular DNA, and reintroduced into the NK-92 cells. This preparation of cells will then express cell surface HLA molecules incorporating mutated β_2 -microglobulin moieties, and will have lost the ability to bind T-cell receptors.

10 **Example 22. NK-92 Cells Expressing Receptors for a Cancer Cell.**

The CTL of a patient suffering from a cancer are to be harvested by differential centrifugation on a density gradient. The CTL are to be immunoaffinity purified to contain predominantly the CTL targeting a receptor on the cancer cell from the patient. The DNA of the CTL population obtained is to be isolated, and the genes for the MHC class I receptor in the cancer-targeted CTL isolated by restriction nuclease cleavage. The genes so purified are to be amplified using the polymerase chain reaction, and the resulting amplified genes incorporated into a vector suitable for the constitutive expression of the genes in NK-92 cells. The vectors are to be transfected into NK-92 cells, and the modified NK-92 cells so obtained are to be selected using, for example, an antibiotic resistance marker incorporated into the vector. The cells so selected are to be cultured to increase their number. They may then be employed to target specifically the cancer cells in the patient, and treat the cancer occurring in the patient either *ex vivo* or *in vivo*.

25 **Example 23. Use of NK-92 Cells to Kill HIV-Infected Cells.** 8E5 is a cell line harboring HIV that produces HIV virions. 8E5L is a corresponding cell line infected with HIV which does not produce virions. In a cytotoxic

activity experiment in which the chromium release assay was used to evaluate activity, the results presented in Table 8 were obtained. In these experiments, A3.01 cells are an uninfected control cell line.

Table 8. Cytotoxic Activity of NK-92 Cells on HIV-Infected Cells.

Target	E:T Ratio	% Cytotoxicity
A3.01	50:1	43
	20:1	51
	5:1	44
	1:1	44
	50:1	43
8E5L	20:1	37
	5:1	44
	1:1	40
	50:1	76
8E5	20:1	69
	5:1	77
	1:1	65

It is seen from Table 8 that 8E5 cells which produce HIV particles elicit a higher cytotoxic activity than do 8E5L cells, which do not produce HIV particles, and higher than control cells. Without wishing to be bound by
 5 theory, it is believed that the anti-viral effect of NK-92 cells is due to factors such as a direct cytotoxic effect, as well as inhibition through MIP-1 α , which is produced by NK-92 cells in high concentrations (Bluman et al, *J. Clin. Invest.* 97, 2722 (1996)). The results indicate that NK-92 cells effectively lyse HIV-producing cells *in vitro*.

NATURAL KILLER CELL LINES AND METHODS OF USE

ABSTRACT

This invention relates to a natural killer cell line termed NK-92 and to NK-92 cell lines that have been modified by transfection with a vector to confer advantageous properties. Additionally, the invention provides an NK-92 cell, an NK-92 cell modified by transfection with a vector conferring advantageous properties, which is unable to proliferate and which preserves the effective cytotoxic activity. The invention provides a modified NK-92 cell line that is transfected with a vector encoding a cytokine that promotes the growth of NK-92 cells. In a significant embodiment, the cytokine is interleukin 2. The invention additionally provides a modified NK-92 cell line that is transfected with a vector that expresses a thymidine kinase gene. The invention further provides a modified NK-92 cell line that is transfected with a vector that expresses a β_2 microglobulin that has lost the ability to bind to T-cell receptors.